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Genetic control of visceral leishmaniasis in a Sudanese population: candidate gene testing indicates a linkage to the NRAMP1 region

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There is some evidence showing that genetic factors are involved in human susceptibility to parasitic diseases such as schistosomiasis and malaria. Studies have shown that the Nramp1 and H-2 genes are implicated in the control of Leishmania donovani infection in mice. We sought genetic loci involved in the control of susceptibility to visceral disease caused by L. donovani in humans. We studied 37 families with at least two affected sibs living in a village in eastern Sudan, where an outbreak of visceral leishmaniasis occurred between 1995 and 2000. The genetic markers located in five chromosomal regions containing candidate genes were typed: 2q35 (NRAMP1), 5q31–q33 (Th2 cytokine cluster), 6p21 (HLA/TNF-α), 6q23 (INFGRI) and 12q15 (INF-γ). Linkage (multipoint lod-score = 1.08; P = 0.01) was observed for the 5′(CA) repeat polymorphism in the NRAMP1 promoter. This suggests that genetic variations of this gene affect susceptibility to visceral leishmaniasis in this population.
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Introduction

Susceptibility to infectious diseases is a complex process that depends on the balance between destruction of the invading organism and immunopathology. Pathogen and host genetic factors play an important role in the control of infection and the development of disease.[1] Different species of *Leishmania* cause clinically distinct diseases including localised cutaneous-, diffuse cutaneous-, mucocutaneous- and visceral-leishmaniasis (VL).[2] However, the severity of the resulting disease is variable among subjects living in the same endemic area. *Leishmania donovani*, the agent of VL in Sudan, may cause asymptomatic, subclinical infection or severe visceral disease.[3] Immunosuppression and to a lesser extent malnutrition[4] increase the risk of VL in humans. Several susceptibility loci have been identified in experimental infections by *L. major*.[5] The mouse locus on chromosome 11, homologous to human chromosome 5q31–q33, contains a number of genes encoding cytokines that are important in the control of the T helper 1/T helper 2 type differentiation and in macrophage responses.[6] The control of the early stages of *L. donovani* infection in mice is linked to a mutation in the transmembrane domain of Nramp1.[7] This mutation also controls susceptibility to *Salmonella typhimurium*,[8] *Mycobacterium bovis*[9] and *Mycobacterium lepraemurium*[10] in mice. Finally the control of the late stages of *L. donovani* infections is linked to the H-2 locus.[11] The relevance of these results for human VL has not yet been established, although ethnic[12,13] and familial factors[3] were shown to affect the clinical outcome of infection. Statistical models have predicted one or two genes to control susceptibility to cutaneous leishmaniasis caused by *L. peruviana* and *L. braziliensis*[14,15] in two different human populations. Blackwell and co-workers demonstrated that polymorphisms in the *TNF-α* and *TNF-β* genes are associated with an increased risk for mucocutaneous leishmaniasis.[16] Polymorphisms at class I and II gene loci are also associated with different clinical forms of cutaneous leishmaniasis.[17]

Our aim was to investigate the genetic control of human susceptibility to VL during an outbreak of VL that occurred in a village in eastern Sudan between 1995 and 2000. We previously reported that environmental factors are important in the early phase of the outbreak, whereas ethnic and familial factors are the main risk factors for VL when considering the whole outbreak.[18] We carried out a genetic study in the same population. Five chromosomal regions containing genes that are good candidates for the control of *L. donovani* infection were analysed by linkage analysis: 5q31–q33, which has

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Table 1 Families included in the linkage analysis

Nb of affected sibs	*Nb of nuclear families*	*With two parents genotyped*	*With one parent genotyped*	*Nb of healthy child genotyped*	*Nb of affected sib-pairs*	*Nb of subjects genotyped*
2	22	18	4	4	22	88
3	8	7	1	1	24	40
4	6	5	1	2	36	37
5	1	1	0	0	10	7
Total	37	31	6	7	92	172

Nb=number.

been linked to susceptibility to *Schistosoma mansoni*[19] and *Plasmodium falciparum*[20,21] infections; 2q35 (*NRAMP1*); 6p21 (HLA/*TNF-α*); 6q23 (*INFGR1*) and 12q15 (*INF-γ*).

Results

The composition of the familial sample retained for the linkage analysis is summarised in Table 1. Twelve subjects and three families were excluded because their marker allele segregation patterns were inconsistent with Mendelian transmission. We determined the genotypes of 172 individuals from 37 families containing two to five affected sibs. The 37 families included 97 affected children, with 92 possible sib-pairs. The mean age of onset was 10 years (range = 3–27 years). All had developed VL during the outbreak: one subject in 1995, 23 in 1996, 54 in 1997, 14 in 1998 and five in 1999. Both parents were genotyped in 31 out of 37 cases. One or more unaffected sibs were included in the analysis for four of the six families in which only one parent was genotyped.

The results of the linkage analysis are shown in Table 2. No evidence of linkage was found with the *IFNGRI*, HLA/*TNF-α* or 5q31–q33 region microsatellite markers (lod-scores <0.14). Higher MLB lod-scores were obtained for markers at the *IFN-γ* and *NRAMP1* gene loci. For the *IFN-γ* locus, the highest lod-score was obtained with D12S92 (lod-score = 0.94; $P = 0.018$), but this finding was not supported by the results of the multipoint analysis with all of the markers typed in this region (maximum multipoint lod-score = 0.58; $P = 0.05$). Significant evidence of linkage was obtained for 5′(CA)n in the *NRAMP1* promoter (lod-score = 1.32; $P = 0.007$), although this polymorphism is not very informative for linkage. Only three alleles were detected at this site (allele 1 = 199 bp; allele 2 = 201 bp; allele 3 = 203 bp), and linkage information could not be obtained for 61 % of parental alleles segregation (information content = 39%). However, information content was raised to 75% in the multipoint analysis combining all *NRAMP1* markers. A similar maximum multipoint lod-score (lod-score = 1.08; $P = 0.01$) was observed in this analysis at the position of 5′(CA)n and thus confirms the results obtained in the two-point analysis. Allele frequencies of the various *NRAMP1* polymorphisms typed in this study are shown in Table 3. None of these polymorphisms were associated with VL. However, the power of the association analysis was considerably reduced as the number of informative families ranged from 3 to 18 depending on the allele tested.

Discussion

Genetic factors involved in the control of *Leishmania* infections were first identified in mice, which provided candidate loci for the study of the genetic factors involved in human susceptibility to VL.[5] Some of the loci identified are also involved in susceptibility to other infections: the 5q31–q33 region controls the infection level by *S. mansoni*[19] and *P. falciparum*[20,21] infections. This region is also involved in susceptibility to asthma[22] and in the control of sera IgGE levels.[23] Furthermore, this locus is involved in the control of T helper 1/T helper 2 subsets differentiation,[6] which is associated with resistance (Th1) and susceptibility (Th2) to *L. major* in inbred strains of mice.[24] Nevertheless, we did not find any linkage with the markers in 5q31–q33. Genetic studies on idiopathic disseminated infection caused by Bacillus–Calmette–Guérin (BCG) or other atypical mycobacteria demonstrated that mutations in molecules of the IFN-γ pathway determine susceptibility to infection. These identified mutations usually have dramatic effects on gene functions and are rare in the general population.[25] Such polymorphisms are unlikely to account for the large number of cases of VL observed in our population. Nevertheless, we observed some linkage (two-point lod-score = 0.94; $P = 0.018$) with microsatellite marker D12S92, which is closely linked to the *IFN-γ* gene. This suggests that even though polymorphisms in this genetic region have a weak effect, they may also affect susceptibility to VL.

An important finding in this study was the nonrandom segregation of the *NRAMP1* gene region among siblings affected with VL. A significant linkage was observed for the 5′(CA) repeat of the *NRAMP1* gene (two-point lod-score = 1.32; $P = 0.007$ and multipoint lod-score = 1.08; $P = 0.01$). This is the first report suggesting that *NRAMP1* alleles are involved in susceptibility to human VL. Different alleles at this polymorphic site have a different effect on the transcription of *NRAMP1 in vitro*.[26] However, in our family-based association analysis, no distinct *NRAMP1* polymorphisms were associated with an increased risk of VL. A much larger sample would probably be necessary to reveal any such association. The lack of association also suggests that the causal polymorphism(s) located in this region is not in strong linkage disequilibrium with the polymorphisms that were typed in this study. It is also possible that the linkage is due to a nearby susceptibility gene. The *NRAMP1* protein is a macrophage-restricted divalent cation transporter, implicated in iron homeostasis.[27] The *NRAMP1* protein is localised in the late endocytic

Table 2 Results of the MLB linkage analysis for the five chromosomal regions tested

Locus (candidate gene) *Markers*	*MLB statistic*			*Marker characteristics*	
	Lod-score	*P*	*Information content (%)*	*Position/gene*	*Polymorphism type*
2q35 (*NRAMP1*):					
5′ (CA)*n*	1.32[a]	0.007	39	Promoter	CA repeat
274 C/T	0.17	n.s.	22	Exon 3	SNP
469+14 G/C	0.05	n.s.	13	Intron 4	SNP
1729+55del4	0.25	0.13	31	3′ UTR	Deletion
D2S1471	0.45	0.1	79	Extragenic	CA repeat
M.M. lod-score[b]	**1.08**	**0.01**	**75**		
5q31–q33 (cytokine cluster)[c]					
D5S471	<0.01	n.s.	68	Extragenic	CA repeat
D5S2057	0.11	n.s.	78	Extragenic	CA repeat
D5S2115	0.08	n.s.	79	Extragenic	CA repeat
D5S436	<0.01	n.s.	67	Extragenic	CA repeat
D5S636	<0.01	n.s.	77	Extragenic	CA repeat
D5S410	<0.01	n.s.	44	Extragenic	CA repeat
D5S422	<0.01	n.s.	75	Extragenic	CA repeat
6p21 (*HLA/TNF-α*)					
D6S276	0.13	n.s.	77	Extragenic	CA repeat
6q23–q24 (*IFNGRI*)					
D6S1009	<0.01	n.s.	85	Extragenic	CA repeat
FA1	<0.01	n.s.	78	Intron	CA repeat
D6S310	<0.01	n.s.	74	Extragenic	CA repeat
12q15 (*IFN-γ*)					
D12S83	<0.01	n.s.	78	Extragenic	CA repeat
JAP	0.26	0.13	53	Intron	CA repeat
D12S92	0.94	0.018	75	Extragenic	CA repeat
D12S326	0.45	0.07	68	Extragenic	CA repeat
M.M. lod-score[b]	**0.58**	**0.05**	**92**		

The maximum multipoint lod-score is given for the two regions (2q35 and 12q15) analysed by multipoint analysis (bold). Information content is the fraction of parental allele segregations that were informative in the analysis.
[a]Two-point MLB lod-score.
[b]Maximum multipoint lod-score over the corresponding chromosomal region.
[c]The 5q31–q33 region contains a cluster of cytokine genes coding for IL-3, IL-4, IL-5, IL-9, IL-12 p40 subunit and other Th1/Th2 related genes: *IRF1, CSF2, CSF1R*.
n.s.=non-significant.

compartment of resting macrophages. After phagocytosis it is recruited to the membrane of the phagosomes.[28] It has been suggested that *NRAMP1* limits the replication of intracellular pathogens by altering the phagolysosomal environment, especially iron concentrations, which are critical for the generation of oxygen free radicals.[29] Together with the results observed in human tuberculosis,[30,31] leprosy[32] and HIV,[33] our data support the view that *NRAMP1* is implicated in the control of intracellular pathogens in humans and in mice. Nevertheless, the strength of the genetic linkage observed in our study was only just significant ($P=0.01$). This suggests that polymorphisms of the *NRAMP1* gene only explain a small part of the familial cases that occurred during the outbreak of VL. A replication study on an independent set of VL multicase families is required to validate the observed linkage at the 2q35 locus. Association studies as performed by the transmission/disequilibrium test have a greater power than linkage analysis to detect disease genes with modest effects.[34] Furthermore, this approach, which requires the ascertainment of single VL patients and their parents, appears more suitable to constitute large sample size. Such studies looking at the *NRAMP1* polymorphisms will be useful to further characterise the role of this gene in human genetic susceptibility to VL.

Our initial goal was to examine candidate genes/gene regions based on known murine susceptibility genes[5] and human loci readily identified in other infectious diseases.[1] Our results provide evidence that the *NRAMP1* gene is involved in susceptibility to VL; however, none of the regions investigated appeared to contain any major genes involved in determining susceptibility to visceral leishmaniasis in this population. The next stage of this genetic analysis will be to perform a genome-wide search, with the aim of identifying chromosomal regions with larger effects.

Patients and methods

Study area and study population

This study was carried out in the village of Barbar El Fugara, located in an agricultural area in eastern Sudan where VL is endemic.[35] An outbreak of VL started in the

Table 3 Association between visceral leishmaniasis and different *NRAMP1* polymorphisms

NRAMP 1 polymorphisms	*Allele frequency*	*Nb of informative families*	*FBAT statistic P-values**
5′ (CA)*n*			
199 bp	0.73	18	0.63
201 bp	0.24	17	0.51
203 bp	0.03	3	—
274 C/T			
C	0.77	12	0.4
T	0.23		
469+12 G/C			
G	0.91	8	1
C	0.09		
3′ UTRdel4			
TGTG +	0.73	15	0.25
TGTG−	0.27		

**P*-values were calculated by the family-based association test (FBAT) program, which tests for transmission disequilibrium in extended multicase nuclear families.

village in 1995. The village was populated by migrants from different ethnic groups (Haoussa, Fellata and Aringa). Only the Aringa (the most affected ethnic group) who migrated from the western Sudan/Chad border area during the 1960s and 1970s were studied to minimise the effect of population admixture. The linkage analysis looked at nuclear families in which at least two sibs were affected. When a parent was not available for genotyping, healthy sibs were included to determine the parental genotype. Blood samples were collected in 1999 from 187 individuals belonging to 40 nuclear families. A total of 105 children from these 40 families were affected with VL during the outbreak.

Ethical considerations

The study protocol, including the protocol for the collection of blood samples, was approved by both the Federal and Gedarif State Ministries of Health and by the Faculty of Medicine, Khartoum University. Informed consent was obtained from the district authorities, from the village committee and from all participating adults. For children, consent was obtained from their parents. Children less than 5-years old were not bled.

Diagnosis and treatment

Lymph node aspirate samples were taken from subjects with clinical signs of VL (as assessed by two physicians) and submitted to parasitological examination. Subjects with both the clinical signs of VL and a positive parasitological examination were considered to be positive for VL and were treated according to WHO protocols.

Investigated chromosomal regions and genotyping

Polymorphism type and genetic location of the genetic markers that were typed in this study are summarised in Table 2. One marker is in the HLA-TNF region (6p21), three in the *IFNGRI* region (6p23–q24), four in the *INF-γ* region (12q15), seven in the 5q31–q33 cytokine cluster region and four in the *NRAMP1* region (2q35). Primers for the amplification of the microsatellites were obtained from the Genethon website (http://www.genethon.fr). Two additional microsatellite markers were also included: FA1, an *IFNGRI* intragenic marker (Genebank accession number U84721[36]), and JAP,[37] an *IFN-γ* intragenic marker. Primer sequences for the *NRAMP1* intragenic polymorphic sites were previously described.[38]

DNA was extracted from blood leukocytes using a standard salting-out method.[39] PCR products from length polymorphism sites were genotyped by use of an ABI 310 sequencer and analysed by Genescan analysis 3.1 and Genotyper 2.1 software (Perkin-Elmer). The single nucleotide polymorphisms (SNPs) were typed following digestion of the PCR products using restriction enzymes as described by Liu *et al*.[38]

Statistical analysis

Linkage analysis was performed using the maximum likelihood binomial (MLB) model-free method.[40] This approach is based on the binomial distribution of parental alleles among affected sibs, and does not need to decompose sibships with more than two affected into constitutive sib-pairs. The linkage test is a simple likelihood ratio test, involving a single parameter that is asymptotically distributed as a 50:50 mixture with 0 and 1 degrees of freedom. The resulting statistic can be expressed as a lod-score, which has the same distribution as a classical model-based lod-score estimating the recombination fraction. Multipoint development of MLB has been implemented[41] in an extension of the GENEHUNTER program.[42] Large simulation studies showed that the MLB test consistently provides type I errors when asymptotic distributions are used, and that the MLB test is generally more powerful than the widely used MLS test, implemented in the MAPMAKER/SIBS program.[40] As five independent regions were tested in the present analysis, a *P*-value of 0.01 was taken as significant evidence for linkage.

The association study was conducted by a family-based approach, to avoid any confounding of gene–phenotype associations due to inappropriately chosen controls or population substructures. The principle of family-based association studies is to search for a distortion of the transmission of candidate gene alleles from parents to affected children by use of the transmission disequilibrium test (TDT).[43] Families with missing parental data can be analysed by either reconstructing parental genotypes from children (RC-TDT)[44] or by using unaffected sibs as controls (Sib-TDT).[45] We used the method implemented in the FBAT program,[46] which combines the three different methods described above (TDT, RC-TDT and Sib-TDT). Furthermore, the FBAT approach allows the use of an empirical variance–covariance estimator, which is consistent when sibling marker genotypes are correlated (eg when there is linkage and the analysis includes multiplex families).[47]

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