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Short communication

Fine mapping under linkage peaks for symptomatic or asymptomatic outcomes of *Leishmania infantum* infection in Brazil

Jason L. Weirather [a,1], Priya Duggal [b,1], Eliana L. Nascimento [c,d], Gloria R. Monteiro [d], Daniella R. Martins [d], Henio G. Lacerda [c,d], Michaela Fakiola [e,2], Jenefer M. Blackwell [e,f,1], Selma M.B. Jeronimo [d,g,h,1], Mary E. Wilson [a,i,j,k,*,1]

[a] Interdisciplinary Program in Genetics, University of Iowa, Iowa City, IA, USA
[b] Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA
[c] Department of Infectious Diseases, Federal University of Rio Grande do Norte, Natal, RN, Brazil
[d] Institute of Tropical Medicine of Rio Grande do Norte, Federal University of Rio Grande do Norte, Natal, RN, Brazil
[e] Cambridge Institute for Medical Research, University of Cambridge, UK
[f] Telethon Kids Institute, The University of Western Australia, Perth, Australia
[g] Department of Biochemistry, Federal University of Rio Grande do Norte, Natal, RN, Brazil
[h] Institute of Science and Technology of Tropical Diseases, Brazil
[i] Department of Internal Medicine, University of Iowa, Iowa City, IA, USA
[j] Department of Microbiology, University of Iowa, Iowa City, IA, USA
[k] Iowa City Veterans' Affairs Medical Center, Iowa City, IA, USA

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ABSTRACT

Infection with the protozoan *Leishmania infantum* can lead to asymptomatic infection and protective immunity, or to the progressive and potentially fatal disease visceral leishmaniasis (VL). Published studies show host genetic background determines in part whether infected individuals will develop a symptomatic or asymptomatic outcome. The purpose of the current study was to fine map chromosome regions previously linked with risk for symptomatic (chromosome 9) or asymptomatic (chromosomes 15 and 19) manifestations of *L. infantum* infection. We conducted a family-based genetic study of VL and asymptomatic infection (detected by a DTH skin test) with a final post quality control sample of 961 individuals with full genotype and phenotype information from highly endemic neighborhoods of northeast Brazil. A total of 5485 SNPs under the linkage peaks on chromosomes 9, 15 and 19 were genotyped. No strong SNP associations were observed for the DTH phenotype. The most significant associations with the VL phenotype were with SNP rs1470217 ($p = 5.9e-05$; $p_{corrected} = 0.057$) on chromosome 9, and with SNP rs8107014 ($p = 1.4e-05$; $p_{corrected} = 0.013$) on chromosome 19. SNP rs1470217 is situated in a 180 kb intergenic region between *TMEM215* (Transmembrane protein 215) and *APTX* (Aprataxin). SNP rs8107014 lies in the intron between exons 26 and 27 of a 34 exon transcript (ENST00000204005) of *LTBP4*, (Latent transforming growth factor-beta-binding protein 4a). The latter supports growing evidence that the transforming growth factor-beta pathway is important in the immunopathogenesis of VL.

1. Introduction

Visceral leishmaniasis (VL) is a debilitating parasitic disease of humans caused by protozoa belonging to the *Leishmania donovani* complex. Symptomatic VL is a severe progressive infection which can be fatal even with treatment. Despite its potential severity, 80–90% of individuals infected with the causative parasites harbor either sub-clinical or asymptomatic infection (Blackwell et al., 2009). The hypothesis that human genetic variants also influence susceptibility to both VL and a positive DTH response is supported by segregation analyses in Brazilian populations (Feitosa et al., 1999; Peacock et al., 2001). Efforts to identify the specific genes conferring susceptibility have inspired candidate gene (reviewed Blackwell, 2010; Blackwell et al., 2009), as well

* Corresponding author at: Internal Medicine-Microbiology, University of Iowa, SW34-GH, 200 Hawkins Dr., Iowa City, IA 52242, USA.
E-mail addresses: jason-weirather@uiowa.edu (J.L. Weirather), pduggal@jhsph.edu (P. Duggal), eltomaz@gmail.com, etomaz@cb.ufrn.br (E.L. Nascimento), gloriag74@hotmail.com (G.R. Monteiro), daniellamartins@cb.ufrn.br (D.R. Martins), heniolacerda@ufrnet.br (H.G. Lacerda), mf300@cam.ac.uk (M. Fakiola), jenefer.blackwell@telethonkids.org.au (J.M. Blackwell), smbj@cb.ufrn.br (S.M.B. Jeronimo), mary-wilson@uiowa.edu (M.E. Wilson).
[1] These authors contributed equally to this work.
[2] Current address: Institute of Molecular Genetics, Milan, Italy.

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as genome-wide linkage (Bucheton et al., 2003; Jamieson et al., 2007; Jeronimo et al., 2007a; Miller et al., 2007) and association (Fakiola et al., 2013) studies. Previously we carried out a genome-wide linkage study (Jeronimo et al., 2007a) that identified a region of putative linkage to symptomatic VL on human chromosome 9, with further regions on chromosomes 15 and 19 identified as carrying loci regulating asymptomatic disease as measured by a delayed type hypersensitivity (DTH) skin test response to crude leishmanial antigen. The purpose of the current study was to fine map these chromosome regions using high density single nucleotide polymorphism (SNP) genotyping and association analyses. The results support growing evidence that the transforming growth factor-beta pathway is important in the immunopathogenesis of VL.

2. Materials and methods

2.1. Subject sample and phenotype

Details of the study site in Natal, Rio Grande do Norte, Brazil, enrollment of subjects, and clinical phenotyping are described in full in our previous genome-wide linkage (Jeronimo et al., 2007a) and candidate gene (Jeronimo et al., 2007b) studies. Briefly, criteria for diagnosis of VL were a clinical presentation with hepatosplenomegaly, fever, cachexia and pancytopenia, positive parasitologic diagnosis (positive bone marrow aspirate, positive serology), and response to treatment. As before (Jeronimo et al., 2007a; Jeronimo et al., 2007b), the cutoff for a positive Montenegro test for *Leishmania* antigen was ≥5 mm of induration. The study was approved by the institutional review boards of the Universidade Federal do Rio Grande do Norte (numbers 19–01 and 21–01); the Comissão Nacional de Ética em Pesquisa (CONEP numbers 4581 and 4575); the University of Iowa; Johns Hopkins University; the University of Virginia; and the National Human Genome Research Institute, National Institutes of Health. Written consent was obtained from adults and from parents or guardians of minors <18 years of age, and written assent was obtained from minors 12–17 years of age.

2.2. Numbers of subjects

DNA for genotyping was available for 1200 individuals (49% male; 51% female), who all contributed to calculation of allele frequencies and linkage disequilibrium (LD) blocks. Full phenotype data was available for 961 genotyped individuals (145 VL; 421 DTH+; 395 DTH−). The study sample comprised 49% males and 51% females.

2.3. SNP selection and genotyping

SNPs (N = 6026) were selected to cover three regions of putative linkage in our prior study (Jeronimo et al., 2007a). Based on our knowledge of admixture in the region of northeast Brazil (Ettinger et al., 2009), tagging SNPs (minor allele frequency > 0.05) were selected from LD blocks using the CEU and YRI populations in HapMap (Table S1). SNP selection was based on >1 SNP per LD block with $r^2 > 0.8$. SNPs between LD blocks were included to ensure coverage. The median distance between the 5485 post quality control (cf. below) SNPs was 10.2 kb. Genotyping was performed by the Center for Inherited Diseases Research at Johns Hopkins University, Baltimore, MD, USA, using the Illumina Infinium genoptyping platform. SNPs with median $p < 0.001$ for deviation from Hardy-Weinberg equilibrium (Wigginton et al., 2005) across unrelated individuals were removed. PEDSTATS (Wigginton and Abecasis, 2005) and MERLIN (Abecasis et al., 2002) software were used to remove Mendelian errors and unlikely genotypes (unlikely recombination events). Individuals or SNPs with >2% inconsistent calls or errors were removed from the analysis. Nuclear families with >5% errors were also excluded. After quality control, 5485 of the original 6026 SNPs were retained in the analysis. The call rate for SNPs among genotyped individuals was 99.91% after quality control.

2.4. Association analyses

Family-based association tests for qualitative traits (VL or DTH positive results) were conducted on all 5485 SNPs using the LAMP software package (Li et al., 2005). The population prevalence for DTH+ was set at 0.7, and at 0.5 for VL, based on observed prevalence in the study population. A modified Bonferroni threshold for significance was calculated to take account of the number of LD blocks identified using a conservative method (Gabriel et al., 2002) implemented in the Haploview program (Barrett et al., 2005). There were 289 blocks on chromosome 9, 355 blocks on chromosome 15 and 317 blocks on chromosome 19. Considering the total of 961 LD blocks, a threshold of $p = 5.2e-05$ (i.e. $p = 0.05/961$) was required to achieve significance at $\alpha = 0.05$. Individual corrected p-values were nominal p-values multiplied by 961 LD blocks. In addition, p-values were calculated separately for each region from permutation tests, using a set of 1000 simulated populations generated with the MERLIN software simulation feature. Simulated data sets maintain the same allele frequencies and missing data points as the original study population. Plots of associations were generated with the Locuszoom software package (Pruim et al., 2010).

3. Results

3.1. Allelic associations

Table S2 lists the most significant associations (uncorrected $p < 0.001$) between each phenotype and markers in linkage regions.

On chromosome 9, SNP rs1470217 had a nominal p value of $p = 5.9e-05$ (empirical simulation $p = 0.089$; Bonferroni corrected $p = 0.057$) for association with a VL outcome (Table 1, Fig. 1, Table S2), with the A allele as the risk allele for VL. Despite the fact that SNP rs1470217 was selected as part of LD block 120 on chromosome 9 (see Table S1), this SNP association was not well supported by other SNPs in strong LD (Fig. 2A) in our study population. Therefore, deeper coverage of SNPs may be required to validate the association in this region. SNP rs1470217 is situated in a 180 kb intergenic region between *TMEM215* (Transmembrane protein 215) and *APTX* (Aprataxin)

Table 1
Locations and allele frequencies of the SNPs most highly associated with the VL phenotype. The number of VL affected individuals for each genotype is shown to provide insight into the possible effect of each allele. p-Values provided are: p_{un} for uncorrected, p_{es} for empirical simulation corrected, p_{corr} for modified Bonferroni correction (i.e. uncorrected p-value multiplied by 961 LD blocks). The overall threshold for significance taking account of the number of LD blocks is $p = 5.2e-05$ (i.e. $p = 0.05/961$ LD blocks) for an $\alpha = 0.05$.

Associated SNP	Position relative to nearest genes	Population allele frequencies	Genotype frequencies	Total (affected) per genotype	p-Values
rs1470217	115 kb downstream of *TMEM215*	A = 0.678	GG = 0.13	GG = 107 (13)	$p_{un} = 5.9e-05$
Chr 9p21.1	69 kb downstream of *APTX*	G = 0.322	AG = 0.45	AG = 299 (78)	$p_{es} = 0.089$
	Intergenic		AA = 0.42	AA = 259 (48)	$p_{corr} = 0.057$
rs8107014	*LTBP4*	C = 0.539	CC = 0.34	CC = 254 (48)	$p_{un} = 1.4e-05$
Chr 19q13.2	Intronic	T = 0.461	CT = 0.5	CT = 331 (63)	$p_{es} = 0.022$
			TT = 0.16	TT = 80 (28)	$p_{corr} = 0.013$