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Genetic and functional evaluation of the role of DLL1 in susceptibility to visceral leishmaniasis in India

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ABSTRACT

Chromosome 6q26–27 is linked to susceptibility to visceral leishmaniasis (VL) in Brazil and Sudan. DLL1 encoding the Delta-like 1 ligand for Notch 3 was implicated as the etiological gene. DLL1 belongs to the family of Notch ligands known to selectively drive antigen-specific CD4 T helper 1 cell responses, which are important in protective immune response in leishmaniasis. Here we provide further genetic and functional evidence that supports a role for DLL1 in a well-powered population-based study centred in the largest global focus of VL in India. Twenty-one single nucleotide polymorphisms (SNPs) at *PHF10/C6orf70*/DLL1/*FAM120B/PSMB1/TBP* were genotyped in 941 cases and 992 controls. Logistic regression analysis under an additive model showed association between VL and variants at DLL1 and *FAM120B*, with top associations (rs9460106, OR = 1.17, 95%CI 1.01–1.35, $P = 0.033$; rs2103816, OR = 1.16, 95%CI 1.01–1.34, $P = 0.039$) robust to analysis using caste as a covariate to take account of population substructure. Haplotype analysis taking population substructure into account identified a common 2-SNP risk haplotype (frequency 0.43; $P = 0.028$) at *FAM120B*, while the most significant protective haplotype (frequency 0.18; $P = 0.007$) was a 5-SNP haplotype across the interval 5′ of both DLL1 (negative strand) and *FAM120B* (positive strand) and extending to intron 4 of DLL1. Quantitative RT/PCR was used to compare expression of 6q27 genes in paired pre- and post-treatment splenic aspirates from VL patients ($N = 19$). DLL1 was the only gene to show differential expression that was higher ($P < 0.0001$) in pre- compared to post-treatment samples, suggesting that regulation of gene expression was important in disease pathogenesis. This well-powered genetic and functional study in an Indian population provides evidence supporting DLL1 as the etiological gene contributing to susceptibility to VL at Chromosome 6q27, confirming the potential for polymorphism at DLL1 to act as a genetic risk factor across the epidemiological divides of geography and parasite species.

1. Introduction

Visceral leishmaniasis (VL) caused by the *Leishmania donovani* complex is endemic in the Indian subcontinent which forms the largest global focus of disease. Only about 10% of individuals infected with *L. donovani* develop clinical disease which is lethal without treatment (Badaro et al., 1986; Topno et al., 2010; Zijlstra et al., 1995). Understanding parasite, host and environmental factors that determine asymptomatic infection versus lethal clinical disease is important in disease control. Host genetic factors are known to contribute to disease susceptibility (reviewed (Blackwell et al., 2009)), and in a recent report (Fakiola et al., 2011) we provided genetic and functional evidence to support DLL1, encoding the Delta-like 1 ligand for Notch 3, as the etiological susceptibility gene at the chromosomal region 6q27 previously linked to susceptibility to VL in both Sudan and Brazil (Jamieson et al., 2007; Miller et al., 2007).

Notch signalling is one of the most conserved pathways regulating cell differentiation/cell fate decisions (Artavanis-Tsakonas et al., 1999; Greenwald, 1998), playing an important role during

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development and in development and differentiation of immune cells. In bone marrow, Notch ligand Delta-1 completely inhibits differentiation of human hematopoietic progenitors into the B cell lineage while promoting emergence of cells with a T cell/natural killer precursor phenotype (Jaleco et al., 2001). Delta-1 also inhibits the differentiation of monocytes into macrophages and promotes their differentiation into dendritic cells (Ohishi et al., 2001). In spleen, Notch signalling is required to maintain CD8 positive dendritic cells (Sekine et al., 2009). Antigen presenting cells, including dendritic cells, also use Notch signalling to promote T helper cell differentiation in response to specific antigens (Amsen et al., 2004). The different Notch ligand families, Delta1,4 versus Jagged1,2, instruct antigen-driven CD4 T cell selection down Th1 versus Th2 fates, respectively (Amsen et al., 2004; Maekawa et al., 2003). The effect of polarized antigen-specific Th1 versus Th2 responses on the outcome of leishmaniasis is well established in murine models (Locksley and Scott, 1991; Scott et al., 1989) and human disease (Pirmez et al., 1993; Sundar et al., 1997). Clinical VL, in particular, has been associated with high Th2 cytokine responses (Sundar et al., 1997), while Th1-generated interferon-γ is higher in children infected with *L. infantum chagasi* that do not progress to clinical VL than those who do (Carvalho et al., 1992). The potential for Delta-1 driven Th1 differentiation to alter the course of infection has already been demonstrated for *L. major* infection in BALB/c mice (Maekawa et al., 2003), making genetic regulation of DLL1 expression a highly plausible explanation for the genetic associations and regulation of splenic expression we previously reported for this 6q27 gene (Fakiola et al., 2011).

The genetic studies undertaken in Sudan and Brazil were limited in terms of sample size and power (Fakiola et al., 2011), making it desirable to determine whether fine mapping of the Chromosome 6q27 region using a well-powered sample would replicate these earlier findings. To achieve this, and to determine the geographical extent of the influence of polymorphism at DLL1 on genetic susceptibility to VL, we turned to the largest global focus of VL in India. Fine mapping of disease association for genes across the 6q27 region using a well-powered population-based sample from India, together with a comparison of gene expression pre- and post-treatment in VL patients, confirms DLL1 as the likely etiological gene determining susceptibility to VL.

2. Materials and methods

2.1. Study population

The study was conducted in the highly endemic district of Muzaffarpur in Bihar State, India. Diagnosis of VL was made on the basis of clinical, parasitological and serological criteria as described (Fakiola et al., 2010; Mehrotra et al., 2011; Sundar and Benjamin, 2003). Further epidemiological and demographic details relating to the study samples and study site are described elsewhere (Mehrotra et al., 2011; Singh et al., 2006). The study was performed using a case-control design with a total of 2019 individuals comprising 990 cases and 1029 controls (Table 1) collected during 2009–2010. Information about caste was recorded as a proxy for population substructure.

2.2. Ethics statement

For collection and use of saliva samples and splenic aspirates in India, informed written consent in Hindi was obtained from all participating individuals and from parents of children under 18 years old. Approval for the study was provided by the Ethical Committee of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. For the use of RNA samples from control spleens from necro organ transplantation healthy organ donors obtained in Sweden, ethical approval was provided by the Regionala Etiska Kommittén, Karolinska Institutet, Stockholm (Regional Ethical Committee, Karolinska Institute, Stockholm).

Table 1
Baseline characteristics of the Indian case-control cohort.

Case-control sample	Number[a]
Cases	958
Male	571
Female	387
Mean age at study encounter ± SD (yr)	31.2 ± 16.7
Range	3–73
Mean age at onset of VL ± SD (yr)	26.8 ± 15.3
Religious group	
Hindu	850
Muslim[b]	108
Controls	1015
Male	570
Female	445
Mean age at study encounter ± SD (yr)	31.8 ± 15.9
Religious group	
Hindu	885
Muslim	130

[a] Numbers are given for the individuals with DNA available for genotyping.
[b] Muslim forms a caste, such that religion is taken into account as a covariate in the caste analysis.

2.3. Genetic studies

Genomic DNA was extracted from saliva using the Oragene technology (DNA Genotek, Ontario, Canada) and SNPs genotyped using Sequenom iPLEX platform (Sequenom, San Diego, CA). A total of 21 SNPs at DLL1 and adjacent genes across the Chromosome 6q27 region were genotyped (Table 2). All SNPs met minimum quality control checks for call rate (>99.5%) across all individuals. Two SNPs were excluded from further analysis: PHF10_rs9371126 showed extreme deviation from HWE in controls and TBP_rs6937840 showed significant difference in missingness between cases and controls. A total of 59 cases and 37 controls for which there was >80% missing genotype data were removed from further analysis, providing a sample of 941 cases and 992 controls for genetic analysis. This cleaned dataset had 93.5–99.9% power for odds ratio 1.5, MAF = 0.1–0.5, $P = 0.01$, and 50–93.1% power for odds ratio 1.3. Allelic and haplotype association tests were performed in PLINK (Purcell et al., 2007) using logistic regression analysis under an additive model. Nominal *P*-values are presented throughout, i.e. without correction for multiple testing. Application of a strict Bonferroni correction for 19 independent ($r^2 < 0.8$) SNPs provides a significance cut-off of $P \leqslant 0.003$ (i.e. $P = 0.05/19$). A less stringent cut-off of $P \leqslant 0.006$ allows for non-independence of SNPs due to LD across the region (Supplementary Fig. 1; $P = 0.05/8$, 4 LD blocks and 4 independent SNPs). Haplotype analyses were undertaken using logistic regression analysis in PLINK (Purcell et al., 2007). Inclusion of caste as a covariate, which we have shown to provide a good surrogate for genetic substructure in genome-wide analyses of a separate sample of 989 cases and 1089 controls in this population (unpublished data), was used to take account of population substructure. Caste was converted into a set of binary dummy variables prior to being included as a covariate in the logistic regression. Permuted *P*-values were also determined in PLINK employing the label-swapping max(*T*) permutation method using the *–mperm* command. Empirical *P*-values (EMP1) were obtained for the most significant risk and protective haplotypes after performing 10,000 permutations. Conditional analyses and a version of stepwise regression based on estimating haplotypic effects (odds ratios) were carried out using the program UNPHASED