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Wound healing genes and susceptibility to cutaneous leishmaniasis in Brazil

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ABSTRACT

Leishmania braziliensis causes cutaneous (CL) and mucosal (ML) leishmaniasis. In the mouse, *Fli1* was identified as a gene influencing enhanced wound healing and resistance to CL caused by *Leishmania major*. Polymorphism at *FLI1* is associated with CL caused by *L. braziliensis* in humans, with an inverse association observed for ML disease. Here we extend the analysis to look at other wound healing genes, including *CTGF, TGFB1, TGFBR1/2, SMADS 2/3/4/7* and *FLII*, all functionally linked along with *FLI1* in the TGF beta pathway. Haplotype tagging single nucleotide polymorphisms (tag-SNPs) were genotyped using Taqman technology in 325 nuclear families (652 CL cases; 126 ML cases) from Brazil. Robust case-pseudocontrol (CPC) conditional logistic regression analysis showed associations between CL and SNPs at *CTGF* (SNP rs6918698; CC genotype; OR 1.67; 95%CI 1.10–2.54; $P = 0.016$), *TGFBR2* (rs1962859; OR 1.50; 95%CI 1.12–1.99; $P = 0.005$), *SMAD2* (rs1792658; OR 1.57; 95%CI 1.04–2.38; $P = 0.03$), *SMAD7* (rs4464148; AA genotype; OR 2.80; 95%CI 1.00–7.87; $P = 0.05$) and *FLII* (rs2071242; OR 1.60; 95%CI 1.14–2.24; $P = 0.005$), and between ML and SNPs at *SMAD3* (rs1465841; OR 2.15; 95%CI 1.13–4.07; $P = 0.018$) and *SMAD7* (rs2337107; TT genotype; OR 3.70; 95%CI 1.27–10.7; $P = 0.016$). Stepwise logistic regression analysis showed that all SNPs associated with CL at *FLI1*, *CTGF*, *TGFBR2*, and *FLII* showed independent effects from each other, but SNPs at *SMAD2* and *SMAD7* did not add independent effects to SNPs from other genes. These results suggest that TGFβ signalling via SMAD2 is important in directing events that contribute to CL, whereas signalling via SMAD3 is important in ML. Both are modulated by the inhibitory SMAD7 that acts upstream of SMAD2 and SMAD3 in this signalling pathway. Along with the published *FLI1* association, these data further contribute to the hypothesis that wound healing processes are important determinants of pathology associated with cutaneous forms of leishmaniasis.

1. Introduction

Leishmania infection is associated with a broad spectrum of clinical phenotypes. Whilst broadly driven by parasite species, many studies have demonstrated that host genetic factors play a part in determining the outcome of infection within each species (reviewed (Blackwell et al., 2009; El-Safi et al., 2006; Lipoldova and Demant, 2006; Sakthianandeswaren et al., 2009)). *Leishmania braziliensis* infection causes cutaneous leishmaniasis (CL) with prolonged time to lesion healing. Pro-inflammatory cytokines, including tumour necrosis factor and interferon-γ, and macrophage activation are important in eventual self-healing, but an exaggerated response is associated with mucosal leishmaniasis (ML) (Bacellar et al., 2002; Castes et al., 1993). This suggests that pathology initiated by the host's immune response, rather than the parasite *per se*, contributes to the clinical phenotype.

A number of studies (Cabrera et al., 1995; Castellucci et al., 2006, 2010; Ramasawmy et al., 2010; Salhi et al., 2008) have reported on the role of polymorphisms at candidate immune response genes (*TNFA*, *SLC11A1*, *CXCR1*, *IL6*, *IL10*, *MCP1*) associated with pro- and anti-inflammatory responses in regulating clinical

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Table 1
Characteristics of collections made during the primary (2000–2004) and secondary (2008–2010) sampling periods.

	Primary sample period			Secondary sample period		
	CL	ML	Leishmaniasis *per se*	CL	ML	Leishmaniasis *per se*
No. cases	250	87	337	402	39	441
Males	128	60	188	219	24	243
Females	122	27	149	183	15	198
Age at disease						
Mean	19.1	30.3	22.4	21.5	26.6	21.9
95%CI	17.1–21.2	25.8–34.7	20.3–24.4	20.1–22.9	20.7–32.4	20.6–23.3
No. nuclear families	–	–	168	–	–	157
Total *N* in families/trios	–	–	767	–	–	764

disease outcome in *L. braziliensis* infection in humans. All of these have also been shown to play a role in wound healing responses (Barrientos et al., 2008; da Silva et al., 2011; De Franco et al., 2007; Galindo et al., 2001; Mori et al., 2011; Pradhan et al., 2011; Thuraisingam et al., 2006; Zaja-Milatovic and Richmond, 2008). The importance of wound healing processes in cutaneous forms of leishmaniasis has also been demonstrated from studies mapping murine susceptibility genes (Sakthianandeswaren et al., 2005, 2009, 2010). In particular, fine mapping in the region of Chromosome 9 in mice (Chromosome 11q24 in humans) identified *Fli1* (Friend leukemia virus integration 1; *FLI1* in humans) as a novel candidate influencing both resistance to *Leishmania major* and an enhanced wound healing response (Sakthianandeswaren et al., 2010). Recently we demonstrated (Castellucci et al., 2011) that polymorphism at *FLI1* is associated with CL caused by *L. braziliensis* in humans, with an inverse association (i.e. association with opposite alleles) observed for ML disease. This was interesting in relation to our previous demonstration that the C allele at the *IL6*-174 G/C promoter polymorphism, which determines low levels of IL-6 release from macrophages, was a risk factor for ML disease (Castellucci et al., 2006). IL-6 is known to increase expression of *FLI1* (Thaler et al., 2011). In the wound healing response, both FLI1 (Nakerakanti et al., 2006) and IL-6 (Gressner et al., 2011) repress connective tissue growth factor (CTGF), and all three genes interact with the transforming growth factor beta (TGFβ) pathway. Here we interrogate further the possible roles of wound healing pathways in cutaneous forms of leishmaniasis caused by *L. braziliensis* by looking for genetic associations with polymorphisms in other wound healing genes, including *CTGF, TGFB1, TGFBR1/2, SMADS 2/3/4/7* and *FLII*, all functionally linked along with *FLI1* in the TGFβ pathway.

2. Materials and methods

2.1. Study site, diagnosis and sample collection

The study was conducted in the area of Corte de Pedra, Bahia, Brazil, where *L. braziliensis* is endemic. Corte de Pedra is composed of 20 municipalities in a rural area previously dominated by Atlantic rain forest, where agriculture now underpins the local economy. For host genetic association studies, two family-based cohorts were collected during two study periods, 2000–2004 and 2008–2010, as reported previously (Castellucci et al., 2006, 2010, 2011). Sample collection for the first cohort was based on ascertainment of index cases of ML from medical records of the Corte de Pedra Public Health Post, and active follow-up to identify and collect all other family members, including those with current or past CL disease. This provided DNA samples (Table 1) from 168 nuclear families that contain 250 CL cases and 87 ML cases. Sample collection for the second cohort was based primarily on incident cases of CL or ML presenting to the health post, with family follow-up to acquire samples from parents and affected siblings, and unaffected siblings if one or both parents were missing. This provided DNA samples (Table 1) from 157 nuclear families that contain 402 CL cases and 39 ML cases. GPS co-ordinates were recorded for all households. Sampling during both study periods was well-matched for geographical location (Supplementary Fig. S1), with the majority of families collected from inland regions where ML is more prevalent than in coastal locations (Schriefer et al., 2009).

The case definition of ML is a characteristic mucosal lesion with either parasitological confirmation or two of the three following criteria: positive delayed-type hypersensitivity test (DTH), positive leishmania serology, and a histopathology suggestive of leishmaniasis. All cases in the current study also responded to antileishmanial therapy. CL is defined as the presence of a single chronic ulcerative lesion at a skin site without evidence of mucosal involvement, without evidence of dissemination to 10 or more sites (disseminated leishmaniasis, not studied here due to low sample size), and confirmed by detection of parasites or a minimum of two of the three criteria listed above. Size of largest cutaneous lesion in mm (average of two diameters measured at right angle) was recorded at diagnosis. Past cases that have been treated in the health post of Corte de Pedra had their diagnoses confirmed from the medical records as matching the same criteria defined above, and all past cases were examined for detection of a characteristic well delimited scar. For the primary collection period, average size of largest lesion for CL patients was determined for 60 CL cases collected in parallel with the families (Castellucci et al., 2006). Between-group differences in lesion size were compared using unpaired *T* tests. Informed consent was obtained from all the participants, and the research was approved by the ethical committee of the Hospital Universitário Professor Edgard Santos, Salvador, Brazil. Demographical, epidemiological and phenotype characteristics of participants in the first family cohort were previously described in full (Castellucci et al., 2006). It is possible that some CL cases in our study could progress to ML disease at a later date. Epidemiological studies show that this will affect <4% of CL patients (Marsden, 1986), thus representing a small reduction in the power of our study to detect CL-specific genetic effects.

2.2. Sample collection and DNA extraction

Blood (8 ml) was taken by venipuncture and collected into dodecyl citrate acid (DCA)-containing vacutainers (Becton Dickinson). Genomic DNA was prepared using the proteinase K and salting-out method (Sambrook et al., 1989).

2.3. Genotyping

Genotyping was performed in Cambridge using Taqman technology for polymorphisms at*CTGF, TGFB1, TGFBR1, TGFBR2, SMAD2, SMAD3, SMAD4, SMAD7*, and *FLII* as presented in Table 2. The cut-off