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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

Table 2
Information on the single nucleotide polymorphisms (SNPs) genotyped for *CTGF*, *TGFB1*, *TGFBR1*, *TGFBR2*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7* and *FLII* in the Brazilian study population.

Gene/SNP	Physical position (Chr:bp)	Alleles[a]	Strand[b]	MAF	Caucasian	Asian	African
CTGF_rs6918698	6:132273257	G > C	+	0.471	0.412	0.500	0.517
CTGF_rs9493150	6:132273991	G > C	+	0.343	0.305	0.122	0.372
CTGF_rs2151532	6:132274730	T > C	–	0.154	0.128	0.279	0.093
TGFB1_rs2241715	19:41856886	G > T	–	0.338	0.283	0.465	0.250
TGFB1_rs1800469	19:41860296	C > T	–	0.313	0.288	0.465	0.208
TGFBR1_rs10760673	9:101878622	G > A	+	0.253	0.204	0.453	0.212
TGFBR1_rs2026811	9:101879263	C > A	+	0.278	0.212	0.488	0.261
TGFBR1_rs334358	9:101910613	G > T	+	0.184	0.217	0.045	0.117
TGFBR2_rs6550005	3:30650064	G > A	+	0.345	0.243	0.169	0.509
TGFBR2_rs4955212	3:30669358	G > A	+	0.248	0.252	0.256	0.208
TGFBR2_rs3081020	3:30687969	T > C	+	0.360	0.336	0.674	0.354
TGFBR2_rs1962859	3:30697909	A > G	+	0.167	0.120	0.488	0.208
TGFBR2_rs934328	3:30707744	A > G	+	0.351	0.336	0.651	0.460
TGFBR2_rs2116142	3:30717661	A > G	+	0.304	0.339	0.456	0.225
SMAD2_rs1792658	18:45382605	T > G	–	0.267	0.186	0.539	0.348
SMAD2_rs1631576	18:45403483	G > A	–	0.419	0.495	0.181	0.442
SMAD3_rs11635145	15:67370121	A > G	+	0.400	0.465	0.560	0.314
SMAD3_rs11631785	15:67379980	T > C	+	0.493	0.433	0.407	0.518
SMAD3_rs1465841	15:67385469	G > C	+	0.443	0.408	0.256	0.534
SMAD3_rs16950687	15:67464013	A > G	+	0.432	0.265	0.343	0.407
SMAD3_rs1052488	15:67486847	T > C	+	0.392	0.261	0.291	0.505
SMAD4_rs12455792	18:48572640	C > T	+	0.430	0.381	0.547	0.341
SMAD4_rs12458752	18:48594104	A > G	+	0.288	0.381	0.523	0.106
SMAD7_rs7226855	18:46454048	C > T	–	0.463	0.473	0.157	0.451
SMAD7_rs4464148	18:46459032	A > G	–	0.245	0.286	0.035	0.168
SMAD7_rs2337107	18:46459323	G > A	–	0.371	0.374	0.390	0.894
FLII_rs2746025	17:18151611	G > T	–	0.284	0.341	0.087	0.177
FLII_rs2071242	17:18154519	A > G	–	0.175	0.239	0.238	0.146
FLII_rs2071241	17:18156541	C > T	–	0.203	0.235	0.453	0.088

Physical positions of markers are given according to Build 37.1 of the human genome. Where available, allele frequencies for the minor allele (MAF) in Brazil are shown for Hapmap Caucasian (CEU), Asian (JPT) and African (YRI) populations.

[a] Major > minor alleles for this Brazilian population.

[b] Alleles given according to strand on which the gene is encoded, except for CTGF where alleles for upstream SNPs rs6918698 and rs9493150 are shown according to the strand on which they were called in SNP DB.

(linkage disequilibrium measured as $r^2 > 0.8$) used to select tag-SNPs was based on HapMap CEU (Caucasian) data. All SNPs were in Hardy Weinberg Equilibrium in genetically unrelated founders of the families (data not shown). Missingness (i.e. failure to score on Taqman assays) ranged from 13.4% (149/1115 individuals available for genotyping) to 33.8% (377/1115) across the 29 SNPs. PEDCHECK (O'Connell and Weeks, 1998) was used to determine Mendelian inconsistencies within families and genotypes for these individuals were set to zero for analysis.

2.4. Statistical analyses

Linkage disequilibrium (LD) was determined using Haploview. Association analyses were performed under additive or genotype-wise models using family-based case-pseudocontrol (CPC) conditional logistic regression analysis (Cordell et al., 2004), where each affected offspring is matched to one to three pseudo-controls that derive from the remaining possible genotypes of the parental mating. OR, 95%CI and *P*-values are calculated using robust conditional logistic regression (rCLOGIT) models employing a robust sandwich estimator of variance and a Wald χ^2 test statistic to control for clustering of trios within pedigrees. A likelihood ratio was used to test for significant differences between additive and genotype-wise models to determine whether dominant or recessive models provided a better fit to the model than an additive model. CPC was implemented in STATA v10.0 (http://www.stata.com/). TDT power approximations (Knapp, 1999) were used to determine power of the families to detect genetic associations. Nominal *P*-values are presented throughout, i.e. without correction for multiple testing. Family-based haplotype TDT was performed using TRANSMIT (Clayton and Jones, 1999).

3. Results

3.1. Characteristics of the samples and power to detect association

Table 1 provides details of the samples used in this study. Sample collections made during two different time periods, 2000–2004 and 2008–2010, were well-matched geographically (Supplementary Fig. S1) and demographically (Table 1). As has been observed previously (Jirmanus et al., 2012), mean size of the largest primary cutaneous lesion was significantly greater in ML compared to CL patients in both primary and secondary sampling periods (Fig. 1). Mean size of primary cutaneous lesion did not differ significantly for ML cases collected in primary compared to secondary sampling periods (Fig. 1). Since CL cases collected during the primary sampling period were historical, data for size of primary lesion was not always available. However, data at diagnosis was available for a parallel set of CL patients collected during the primary sampling period (see control CL sample reported in previous genetic study (Castellucci et al., 2006)). A significant difference was observed for mean size of largest lesion for these CL patients compared to those collected during the secondary sampling period (Fig. 1). Parallel epidemiological studies interpret this difference as being due to changes in health seeking behaviour and clinical management which allowed earlier diagnosis and treatment of disease during the second sampling period compared to the primary sampling period (Jirmanus et al., 2012).

Table 2 provides details of SNPs genotyped in this Brazilian population. The lowest minor allele frequency (MAF) was 0.154. Inspection of pairwise LD data (Supplementary Fig. S2) confirmed that 27 of the 29 SNPs genotyped were in linkage equilibrium, as defined by the cut-off ($r^2 < 0.8$) used to select tag-SNPs. Hence,

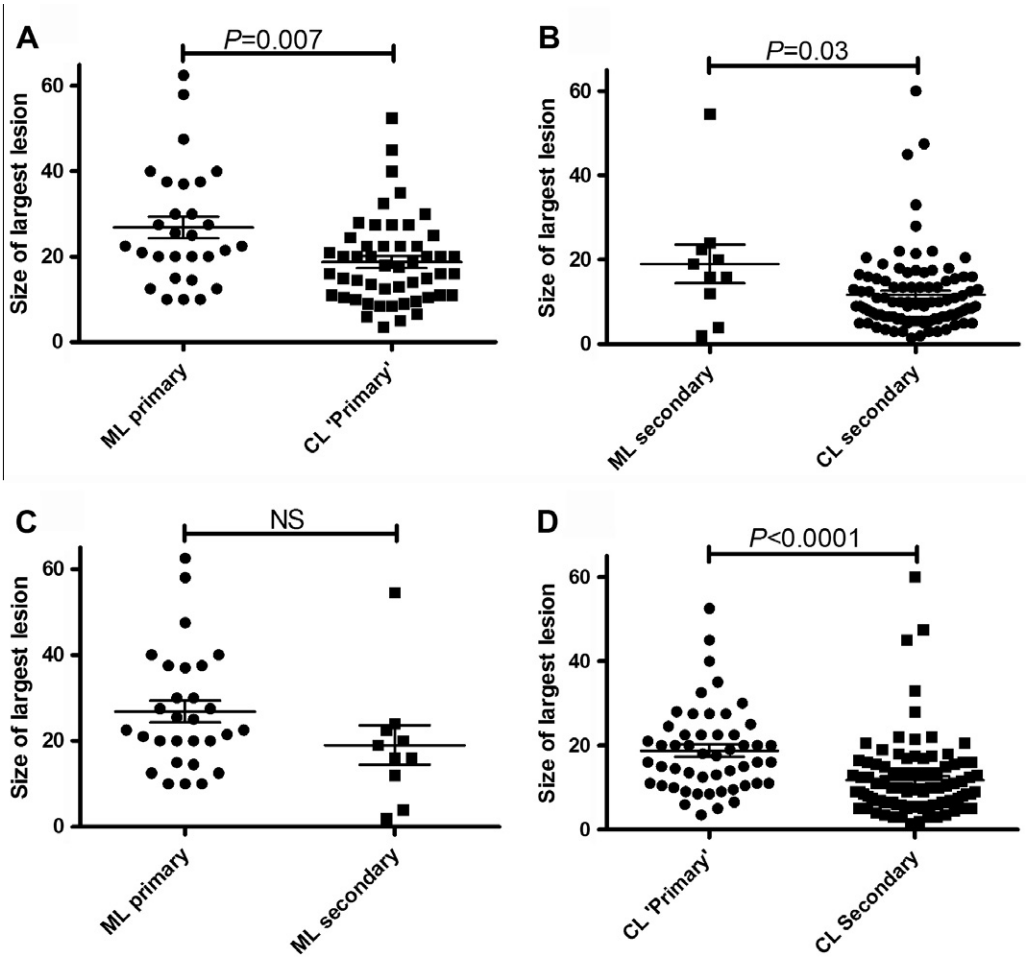

Fig. 1. Mean size of largest cutaneous lesion in ML compared to CL cases for (A) the primary collection period, and (B) the secondary collection period. Graphs (C) and (D) compare ML cases and CL cases across primary and secondary collection periods, respectively. Bars indicate results of between group *T* tests.

the *P*-value needed to achieve statistical significance taking multiple testing into account is $P = 0.002$ (=0.05/27). TDT power approximations (Knapp, 1999) showed that the first (250 CL cases) and second (402 CL cases) family datasets had 53% and 76% power, respectively, to detect an odds ratio $\geqslant 1.5$ at $P = 0.002$ for markers with minor allele frequency (MAF) $\geqslant 0.15$. In contrast, the combined 652 CL trios had 94% power to detect association for the same effect size, *P*-value and MAF. Similarly, for leishmaniasis *per se* (i.e. CL + ML), power estimations for 337 primary, 441 replication, and 778 combined trios had 81%, 81%, and 97% power, respectively. Since the two sample sets were well-matched geographically (Supplementary Fig. S1) and demographically (Table 1), power to detect genetic associations with clinical phenotype (CL, ML or the two combined as leishmaniasis *per se*) was maximised by analysing the combined dataset. Power to detect associations with ML disease was low ($\leqslant$20%) even in the combined dataset.

3.2. Differing patterns of associations between wound healing genes and CL versus ML disease

Results of the CPC logistic regression analysis for the three clinical phenotypes (CL, ML and leishmaniasis *per se*) are presented in Table 3. Only data for SNPs for which a nominal $P \leqslant 0.05$ was observed for at least one clinical phenotype are shown. None of the associations achieves the *P*-value required ($P = 0.002$) to apply a strict correction factor for the 27 independent SNPs genotyped. Nevertheless, a pattern emerges in which the only SNPs showing positive disease associations (nominal $P < 0.05$) in ML cases (*SMAD3* rs1465841 and *SMAD7* rs2337107) are those that are not associated with CL or leishmaniasis *per se*. The possible significance of this is examined below in relation to the role of these genes in TGFβ pathway signalling (Fig. 2).

3.3. Relating associations to role of FLI1

In previous studies we demonstrated association between CL in this study population and polymorphisms at *FLI1* (Castellucci et al., 2011). Therefore, we were interested to determine whether there were further associations at genes encoding molecules known to be directly or indirectly influenced by FLI1 function. Fli-1 acts as a repressor of *CTGF*, competing with Ets-1 in a Sp-1 (Nakerakanti et al., 2006) or Sp-3 (Fonseca et al., 2007) dependent balance between stimulating and repressing gene expression. All 3 tag-SNPs at *CTGF* examined here showed association with CL and leishmaniasis *per se*. SNP rs6918698 lies −945 bp upstream of *CTGF* and is a known functional variant (Fonseca et al., 2007). Substitution of cytosine (C allele) for guanine (G allele) at this site creates a binding site for the transcriptional regulators Sp-1 and Sp-3 (Fonseca et al., 2007). In our study the C allele is the risk factor for CL and leishmaniasis *per se* (Table 3). The C allele binds Sp-3 with high affinity to repress *CTGF* expression (Fonseca et al., 2007). Hence we conclude that repression of CTGF is a risk factor for CL, and that

Table 3
Results of robust case/pseudo-control (CPC) association analysis undertaken to look for association between tag SNPs at wound healing genes (see Table 2) and CL, ML or *L. braziliensis per se* (CL and ML) disease phenotypes in Brazil. # Case/pseudo-control sets equates to number of informative transmissions; OR, odds ratio; CI, 95% confidence interval. Results shown only for SNPs where nominal $P<0.05$ was observed for at least one clinical phenotype.

Gene/marker	Pheno-type	Model	Allele/genotype[a]	# Case/pseudo-control sets	OR	95%CI	*P*-value
CTGF_rs6918698	CL	Genotype	CC	271	1.67	1.10–2.54	**0.016**
CTGF_rs9493150	CL	Additive	G	281	1.31	1.01–1.70	**0.039**
CTGF_rs2151532	CL	Genotype	CC	291	3.17	1.26–7.96	**0.014**
FLII_rs2071242	CL	Additive	T	268	1.60	1.14–2.24	**0.005**
TGFBR2_rs1962859	CL	Additive	A	295	1.50	1.12–1.99	**0.005**
SMAD2_rs1792658	CL	Additive	T	210	1.57	1.04–2.38	**0.030**
SMAD3_rs1465841	CL	Additive	G	290	–	–	NS
SMAD7_rs4464148	CL	Genotype	AA	278	2.80	1.00–7.87	**0.051**
SMAD7_rs2337107	CL	Genotype	TT	282	–	–	NS
CTGF_rs6918698	ML	Genotype	CC	49	–	–	NS
CTGF_rs9493150	ML	Additive	G	49	–	–	NS
CTGF_rs2151532	ML	Genotype	CC	51	–	–	NS
FLII_rs2071242	ML	Additive	T	50	–	–	NS
TGFBR2_rs1962859	ML	Additive	A	54	–	–	NS
SMAD2_rs1792658	ML	Additive	T	32	–	–	NS
SMAD3_rs1465841	ML	Genotype	G	52	2.15	1.13–4.07	**0.018**
SMAD7_rs4464148	ML	Genotype	AA	53	–	–	NS
SMAD7_rs2337107	ML	Genotype	TT	50	3.70	1.27–10.7	**0.016**
CTGF_rs6918698	CL + ML	Genotype	CC	320	1.50	1.00–2.25	**0.049**
CTGF_rs9493150	CL + ML	Additive	G	329	1.29	1.03–1.63	**0.026**
CTGF_rs2151532	CL + ML	Genotype	CC	341	2.94	1.24–6.96	**0.014**
FLII_rs2071242	CL + ML	Additive	T	317	1.55	1.15–2.09	**0.003**
TGFBR2_rs1962859	CL + ML	Additive	A	348	1.35	1.05–1.75	**0.019**
SMAD2_rs1792658	CL + ML	Additive	T	242	1.61	1.11–2.35	**0.012**
SMAD3_rs1465841	CL + ML	Additive	G	341	–	–	NS
SMAD7_rs4464148	CL + ML	Genotype	AA	330	2.80	1.16–6.81	**0.023**
SMAD7_rs2337107	CL + ML	Genotype	TT	331	–	–	NS

Bold indicates $P \leqslant 0.05$.
[a] Significant likelihood ratio test supports recessive model for disease allele.

CTGF is needed to assist in the wound healing process in leishmaniasis.

SNP rs9493150 lies at −1473 bp upstream of *CTGF*, and therefore upstream of rs6918698, with which it is in moderate LD ($D' = 0.95$; $r^2 = 0.43$; Supplementary Fig. S2). Stepwise analysis (Table 4A) shows that addition of SNP rs9493150 does not add significantly to a model in which SNP rs6918698 is considered alone, nor does SNP rs6918698 does add significantly to a model in which SNP rs9493150 is considered alone. Therefore both are tagging the same genetic effect, which is likely to be the known functional

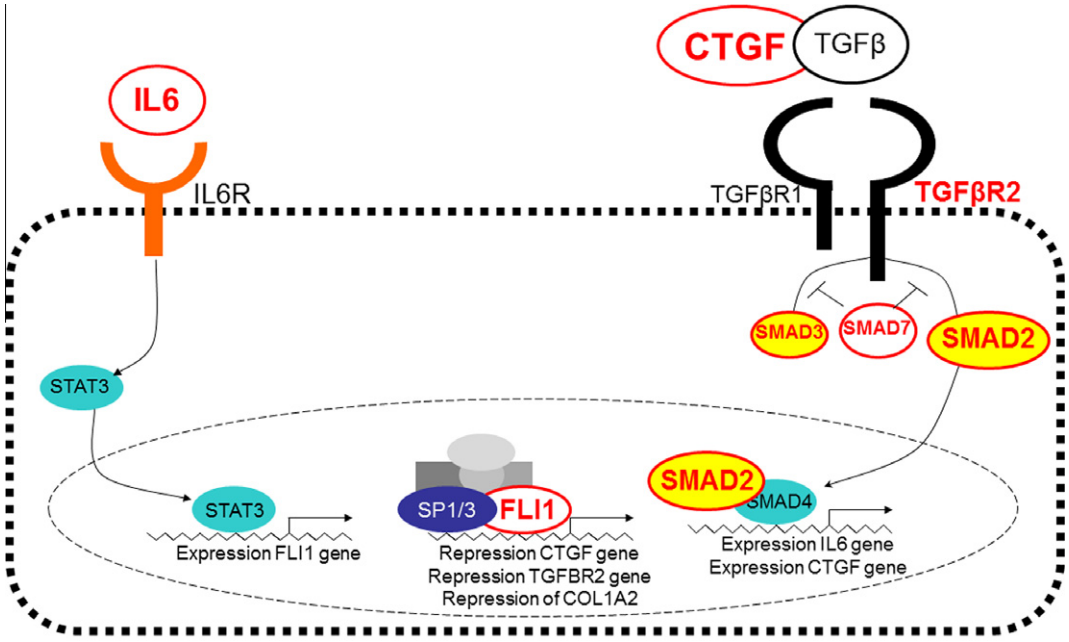

Fig. 2. Diagram of genes that have been implicated in susceptibility to CL and ML disease caused by *L. braziliensis* in the area of Corte de Pedra, Bahia, Brazil, showing involvement of, and interaction with, the TGFβ pathway. Polymorphisms in genes annotated in red lettering have been associated with CL or ML disease.

Table 4
Results of robust (A) intra-locus and (B) inter-locus robust stepwise conditional logistic analyses for SNPs at *CTGF* and *FLI1* to determine independent effects for the CL phenotype in Brazil. Similar results were obtained for *L. braziliensis per se* (CL and ML) (data not shown). χ^2 = Chi-squared; df = degrees of freedom.

Null model	Alternative model	Robust test		
		χ^2	df	*P*-value
(A)				
CTGF_rs6918698	CTGF_rs6918698 + CTGF_rs2151532	4.23	2	0.121
CTGF_rs2151532	CTGF_rs2151532 + CTGF_rs6918698	3.9	2	0.142
CTGF_rs9493150	CTGF_rs9493150 + CTGF_rs6918698	1.88	2	0.390
CTGF_rs6918698	CTGF_rs6918698 + CTGF_rs9493150	0.93	1	0.335
CTGF_rs2151532	CTGF_rs2151532 + CTGF_rs9493150	**4.05**	**1**	**0.044**
CTGF_rs9493150	CTGF_rs9493150 + CTGF_rs2151532	**8.65**	**2**	**0.013**
(B)				
FLI1_rs7930515	CTGF_rs6918698 + FLI1_rs7930515	**13.07**	**1**	**0.0003**
CTGF_rs6918698	FLI1_rs7930515 + CTGF_rs6918698	**7.14**	**2**	**0.028**

Bold indicates $P < 0.05$.

activity of the more strongly associated SNP rs6918698. This is consistent with haplotype analysis showing trends for over-transmission (chi-squared = 3.77; $P = 0.052$) of the common (frequency = 0.45) C_G haplotype across these two SNPs, and under-transmission (chi-squared = 3.65; $P = 0.056$) of haplotype G_C (frequency 0.35), to affected individuals.

SNP rs2151532 lies 2212 bp upstream of CTGF, and is in linkage equilibrium with the above two SNPs ($r^2 < 0.20$; Supplementary Fig. S2). Stepwise analysis shows that addition of SNP rs2151532 to a model that includes rs9493150 adds significantly to a model in which this SNP is considered alone, and the reverse holds true. However, since neither adds to a model in which rs691698 is considered alone, both are likely to be independently tagging the same functional variant with rs6918698. Since rs6918698 is a known functional variant, this SNP was taken forward in inter-locus stepwise conditional logistic regression analyses. This inter-locus stepwise analysis (Table 4B) demonstrates that SNP rs6918698 adds independent effects when compared to a model including the top associated SNP (rs7930515) at *FLI1* shown by us previously to be associated with CL and leishmaniasis *per se* in the same study cohort (Castellucci et al., 2011), and the reverse holds true. Therefore, polymorphisms at *CTGF* and *FLI1* are having independent main effects on susceptibility to CL disease (and to leishmaniasis *per se*, data not shown).

3.4. Relating associations to TGFB signalling

Results above indicate that CTGF is an important molecule in wound healing for CL. Previous studies (Klass et al., 2009) show that TGFβ up-regulates CTGF mRNA. We therefore looked for additional genetic associations between CL and polymorphisms in genes encoding molecules involved in the TGFβ signalling pathway (Fig. 2). TGFβ ligates type I and type II serine threonine kinase receptors, establishing signalling via SMAD2/4- and SMAD3-dependent pathways. SMAD7 acts as a negative regulator of these two SMAD signalling pathways. In our study we did not see associations for tag-SNPs at *TGFB* or *TGFBR1*, but one SNP at *TGFBR2* (rs1962859) was associated with CL and leishmaniasis *per se*. FLI1 is also known to act as a repressor of *TGFBR2* (Hahm et al., 1999). Stepwise conditional logistic regression analysis (Table 5A) demonstrates that *TGFBR2*_rs1962859 adds independent effects when compared to models containing SNPs at *FLI1* or *CTGF*, and the reverse holds true. We also observed association between CL and *SMAD2*_rs1792658, but not between CL and tag SNPs at *SMAD4* or *SMAD3*. Genetic association at *SMAD2*_rs1792658 added independent effects compared to *FLI1*, *CTGF* and *TGFBR2* SNPs (Table 5B), but the reverse does not hold true for *CTGF*. This suggests that effects at *CTGF* are only observed on the background of specific alleles at *SMAD2*, indicating the potential importance of TGFβ signalling via SMAD2 for induction of CTGF.

Association was also observed between CL and SNP rs4464148 in the gene encoding the negative regulator of TGFβ signalling SMAD7. However, this association did not show independent effects (Table 6) when compared to SNPs at *FLI1*, *CTGF*, *TGFBR2* or *SMAD2*, suggesting that effects at *SMAD7* SNP rs4464148 for CL are only observed on the background of specific alleles at each of the other associated genes. Of interest, a second *SMAD7* SNP rs2337107 was associated with ML but not CL disease, as was SNP rs1465841 at *SMAD3*. LD between *SMAD7* SNPs rs2337107 and rs4464148 shows high D' (0.97) but low r^2 (<0.20; Supplementary Fig. S2), suggesting that effects of these SNPs on different forms of disease are caused by different functional variants. The associations with different genes/SNPs in ML compared to CL suggest that TGFβ signalling via SMAD3 is important in ML disease, whereas the SMAD2 signalling pathway regulates gene transcription in CL disease. SMAD7 acts as a negative regulator upstream of both SMAD2 and SMAD3 in the TGFβ signalling pathway.

Table 5
Results of robust intra-locus stepwise conditional logistic analyses for SNPs at (A) *TGFBR2*, *CTGF* and *FLI1*, and (B) *SMAD2*, *TGFBR2*, *CTGF* and *FLI1*, to determine independent effects for the CL phenotype in Brazil. Similar results were obtained for *L. braziliensis per se* (CL and ML) (data not shown). χ^2 = Chi-squared; df = degrees of freedom.

Null model	Alternative model	Robust test		
		χ^2	df	*P*-value
(A)				
TGFBR2_rs1962859	TGFBR2_rs1962859 + CTGF_rs6918698	**9.58**	**2**	**0.008**
CTGF_rs6918698	CTGF_rs6918698 + TGFBR2_rs1962859	**13.54**	**1**	**0.0002**
TGFBR2_rs1962859	TGFBR2_rs1962859 + FLI1_rs7930515	**12.36**	**1**	**0.0004**
FLI1_rs7930515	FLI1_rs7930515 + TGFBR2_rs1962859	**6.07**	**1**	**0.014**
(B)				
SMAD2_rs1792658	SMAD2_rs1792658 + TGFBR2_rs1962859	**7.04**	**1**	**0.008**
TGFBR2_rs1962859	TGFBR2_rs1962859 + SMAD2_rs1792658	**5.42**	**1**	**0.019**
SMAD2_rs1792658	SMAD2_rs1792658 + FLI1_rs7930515	**18.09**	**1**	**0.00002**
FLI1_rs7930515	FLI1_rs7930515 + SMAD2_rs1792658	**10.43**	**1**	**0.0012**
SMAD2_rs1792658	SMAD2_rs1792658 + CTGF_rs6918698	4.49	2	0.1061
CTGF_rs6918698	CTGF_rs6918698 + SMAD2_rs1792658	**4.08**	**1**	**0.0435**

Bold indicates $P < 0.05$.

Table 6
Results of robust inter-locus stepwise conditional logistic analyses for SNPs at SMAD7 compared to *FLI1*, *CTGF*, *TGFBR2*, *SMAD2* and *FLII* to determine independent effects for the CL phenotype in Brazil. Similar results were obtained for *L. braziliensis per se* (CL and ML) (data not shown). χ^2 = Chi-squared; df = degrees of freedom.

Null model	Alternative model	Robust test		
		χ^2	df	*P*-value
SMAD7_rs4464148	SMAD7_rs4464148 + FLI1_rs7930515	**11**	**1**	**0.0009**
FLI1_rs7930515	FLI1_rs7930515 + SMAD7_rs4464148	3.8	2	0.149
SMAD7_rs4464148	SMAD7_rs4464148 + CTGF_rs6918698	**7.21**	**2**	**0.027**
CTGF_rs6918698	CTGF_rs6918698 + SMAD7_rs4464148	3.86	2	0.145
SMAD7_rs4464148	SMAD7_rs4464148 + TGFBR2_rs1962859	**12.07**	**1**	**0.0005**
TGFBR2_rs1962859	TGFBR2_rs1962859 + SMAD7_rs4464148	4.87	2	0.088
SMAD7_rs4464148	SMAD7_rs4464148 + SMAD2_rs1792658	**6.95**	**1**	**0.008**
SMAD2_rs1792658	SMAD2_rs1792658 + SMAD7_rs4464148	4.65	2	0.098
SMAD7_rs4464148	SMAD7_rs4464148 + FLII_rs2071242	**4.42**	**1**	**0.036**
FLII_rs2071242	FLII_rs2071242 + SMAD7_rs4464148	5.09	2	0.078

Bold indicates $P < 0.05$.

3.5. Associations at FLII

The actin remodelling protein Flightless I (FLII) is also an important mediator of wound repair associated with modulation of expression of TGFβ and SMAD 2/3 (Kopecki et al., 2011). We therefore looked for genetic associations between CL and SNPs at *FLII*. Association was observed between tag-SNP rs2071242 at *FLII* and both CL and leishmaniasis *per se*, but not ML disease (Table 3). Effects at this SNP were independent of effects at SNPs in *FLI1*, *CTGF*, *TGFBR2*, *SMAD2* and *SMAD7* (data not shown), but SNP rs4464148 at *SMAD7* did not show independent effects compared to the *FLII* SNP for CL (Table 6) or leishmaniasis *per se* (data not shown).

4. Discussion

Genetic association studies are a powerful way of dissecting out important pathways involved in pathogenesis of disease. Over the last several years data have accumulated on genetic associations that contribute to CL and ML forms of disease caused by *L. braziliensis*. Whilst initial interest focused on genes involved in pro- and anti-inflammatory immune responses to infection, attention has now focused on the complex interaction between immune response genes and genes involved in wound healing. This follows on from functional studies (Sakthianandeswaren et al., 2005) showing that ability to cure cutaneous lesions caused by *L. major* correlated with a wound healing response that presented in congenic resistant mice as a large population of fibroblasts and an organised and abundant deposition of collagen bundles in the absence of inflammatory cells. In contrast, response to wounding in congenic susceptible mice was associated with a larger population of acute inflammatory cells with sparse and disorganised collagen bundles. Genetic studies showed that the *lmr2* gene controlling cutaneous lesions caused by *L. major* on murine Chromosome 9 mapped to the *Fli1* gene (Sakthianandeswaren et al., 2010). We recently demonstrated that polymorphisms at *FLI1* are associated with CL disease caused by *L. braziliensis* in humans (Castellucci et al., 2011), adding to previous studies (Cabrera et al., 1995; Castellucci et al., 2006, 2010; Ramasawmy et al., 2010; Salhi et al., 2008) reporting genetic associations for CL or ML disease at immune response genes (*TNFA*, *SLC11A1*, *CXCR1*, *IL6*, *IL10, MCP1*) associated with pro- and anti-inflammatory responses. All of these are also known 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).

To pursue further the hypothesis that wound healing processes are important in determining disease pathogenesis, we expanded our search for genes involved in CL or ML disease caused by *L. braziliensis* to look at genes known to contribute to the wound healing process through interaction with FLI1 and the TGFβ signalling pathway. The transcription factors Ets1 and Fli-1 have opposing effects on activation and repression of *CTGF*, and induction of the profibrotic gene program (Nakerakanti et al., 2006). TGFβ up-regulates CTGF mRNA (Klass et al., 2009), while FLI1 also acts as a repressor of TGFBR2 (Hahm et al., 1999). Epigenetic repression of the *FLI1* gene is associated with enhanced type I collagen expression (Wang et al., 2006). Hence, there is a complex interplay between FLI1 and the TGFβ signalling pathway in regulating collagen deposition and fibrosis during the wound healing process. In looking for genetic associations that might throw light on wound healing processes important in CL versus ML disease caused by *L. braziliensis*, our results indicate that CTGF regulated via the SMAD2 arm of the TGFβ signalling pathway is required for wound healing in CL disease. In contrast, ML disease was associated with polymorphism in *SMAD3*, suggesting that alternative regulation of gene expression via the TGFβ signalling pathway may lead to ML disease. Further functional data will be required to determine the downstream events following signalling via SMAD3 in ML compared to signalling via SMAD 2 for CL disease might be. Both forms of disease were influenced by polymorphisms in the negative regulator SMAD7 that blocks the TGFβ pathway upstream of both SMAD2 and SMAD3.

Abnormal TGFβ regulation and function are implicated in a large number (reviewed (Santibanez et al., 2011)) of fibrotic and inflammatory pathologies, including pulmonary fibrosis, liver cirrhosis and systemic sclerosis. TGFβ is an important regulator of both physiological fibrogenesis and pathological fibrosis. The fibrotic reaction leads to increased production of extracellular matrix proteins such as fibronectin and collagens, as well as the activation of local fibroblasts to differentiate into myofibroblasts. CTGF expression induced by TGFβ is an important effector of fibrosis, promoting collagen synthesis and myofibroblast differentiation (Pohlers et al., 2009). In contrast, a strong pro-inflammatory response supported by immune studies (Bacellar et al., 2002; Castes et al., 1993) and a number of genetic associations (Cabrera et al., 1995; Ramasawmy et al., 2010), along with alternative regulation of the CTGF/TGFβ pathway via SMAD3 (this study) and IL-6 (Castellucci et al., 2006), appears to accompany ML disease. The importance of the CTGF and the TGFβ pathway in wound healing and pathological fibrosis has already made them attractive targets for therapeutic interventions (reviewed (Asano, 2010; Santibanez et al., 2011)). Our data suggest that repression of CTGF is a risk

factor for CL disease, and therefore that the normal physiological regulation of the wound healing process is important for lesions to heal. In the mouse, differential regulation of Ctgf and collagens (Col1a2, Col18a1, Col1a1) was observed in macrophages from congenic resistant and susceptible mice with and without infection (Sakthianandeswaren et al., 2005), suggesting that macrophages may also play an important role in initiating the profibrotic gene program. Further functional studies will be important to examine expression of members of this important pathway, including the regulation of FLI1 and CTGF in particular, during the natural evolution of disease. This will permit us to determine whether modulation of this pathway through therapeutic intervention using any of the current drugs in clinical trials (see NIH webpage http://clinicaltrial.gov) might have application in some forms of leishmaniasis.

In summary, data presented in this study provide further support for the role of wound healing genes in general, and the pathways regulated by the TGFβ pathway in particular, in determining different clinical phenotypes of disease caused by *L. braziliensis* infection in humans.

Authors' contributions

LC carried out the field collection and preparation of the samples, performed the genotyping, and participated in the statistical analysis and interpretation of the data. S.E.J., M.F. and E.N.M. trained L.C. in the laboratory for genotyping techniques, in database entry and use of the genetic database GenIE in Cambridge, and in genetic statistical analysis methods. S.E.J. and M.F. cross-checked statistical analyses and carried out additional statistical tests. L.A., J.O. and L.H.G. participated in the field collection of data, processing of DNA samples and database entry in Brazil. M.L. is the doctor responsible for confirmation of the M.L. cases by performing ENT exams. A.R.J. trained the field group, initial selection of cases from the health post, assisted with field collection of data and participated in the design of the study. E.M.C. helped conceive the study, initial selection of cases from the health post, and provided the logistical support to make the study possible. J.M.B. participated in the design of the study, conceived the specific hypothesis to be tested, made the final interpretation of the data, and prepared the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.03.017.

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