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SHORT REPORT

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IL-10 promoter single nucleotide polymorphisms are significantly associated with resistance to leprosy

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Abstract The minor haplotype −3575A/-2849G/-2763C in IL-10 promoter has been defined as a marker of disease resistance to leprosy and its severity in Brazilian population. Our investigation of six single-nucleotide polymorphisms (SNPs) in IL-10 promoter in 282 Indian leprosy patients and 266 healthy controls by direct PCR sequencing, however, showed that the extended haplotype: −3575T/-2849G/-2763C/-1082A/-819C/-592C was associated with resistance to leprosy per se and to the development of severe form of leprosy, using either a binomial (controls vs cases, $P = 0.01$, OR = 0.58, CI = 0.37–0.89) or ordinal (controls vs paucibacillary vs multibacillary, $P = 0.004$) model. Whereas, IL-10 haplotype −3575T/-2849G/-2763C/-1082A/-819T/-592A was associated with the risk of development of severe form of leprosy ($P = 0.0002$) in contrast to the minor risk haplotype −3575T/-2849A/-2763C in the Brazilian population. The role of IL10 promoter SNPs in Brazilian and Indian population strongly suggests the involvement of IL-10 locus in the outcome of leprosy.

Introduction

Leprosy, a chronic mycobacterial infection caused by *Mycobacterium leprae* is characterized by clinically defined polar forms (Ridley and Jopling 1966). Patients with tuberculoid leprosy exhibit paucibacillary (PB) infection and a strong cellular immune response, whereas the lepromatous leprosy patients manifest multibacillary (MB) infection and an absence of antigen-specific cellular immunity. The limited genetic diversity between *M. leprae* isolates (Monot et al. 2005) and the clinical spectrum observed among individuals who develop leprosy infection illustrates the significance of host factors in influencing the outcome of infection. Several genomic regions have been implicated in susceptibility to leprosy or severity of the disease (Fitness et al. 2002). Although susceptibility to leprosy has been strongly associated with chromosome 6q25-encoded parkin and parkin co-regulated gene (Mira et al. 2004), however, it is most likely that the genetic control of susceptibility to leprosy is influenced by many different loci.

Cytokines constitute an important group of candidate genes which critically influence host-pathogen interaction and IL-10 emerges as a potent anti-inflammatory and immunosuppressive cytokine, regulating protective immunity in leprosy. IL-10 is enhanced in MB form of the disease (Misra et al. 1995) and a higher TNF/IL-10 ratio is correlated with better prognosis in terms of clinical outcome of leprosy in household contacts (Lima et al. 2000). Single nucleotide polymorphisms (SNPs) and microsatellites in the promoter region of IL-10, form several haplotypes that are associated with differential levels of IL-10 production (Eskdale et al. 1999). The mosaic of distal and proximal IL-10 promoter elements are known to differentially regulate IL-10 secretion (Mormann et al. 2004).

The ethnic differences in distribution of diverse IL-10 gene polymorphisms are an important factor in determining gene–disease association (Opdal 2004; Moraes et al. 2003). IL-10 promoter SNPs have been defined as markers for leprosy susceptibility and severity in Brazilian population (Moraes et al. 2004). India carries the majority of the global burden of leprosy, however, information with respect to the status of IL-10 promoter SNPs is not available in Indian leprosy patients. We carried out a case control association study to assess the influence of IL-10 SNPs; T-3575A, G-2849A, C-2763A,

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A-1082G, C-819T, and C-592A in susceptibility to leprosy or leprosy type.

Materials and methods

A total of 282 leprosy patients from the Hansen's outpatient unit of Lok Nayak Jai Prakash Hospital (New Delhi, India) were included in the study. Diagnosis was made by at least two independent and experienced leprologists following physical examination of each patient and standard histopathologic examination of affected skin lesions. The classification of the patients was based on clinical and histological criteria (Ridley and Jopling 1966). Patients were classified as MB (with bacterial index, BI > 0; $n = 144$), a group that included patients with lepromatous (LL) ($n = 61$), BL ($n = 52$) and borderline (BB) ($n = 31$) leprosy, or as PB (with BI of 0; $n = 142$); patients with borderline tuberculoid (BT) ($n = 141$) and tuberculoid (TT) ($n = 1$) leprosy. Paucibacillary leprosy was characterized by the presence of large well-defined skin lesions which were less than 5 in number, dry and with almost 90–100% loss of sensation. Multibacillary leprosy was characterized by the presence of 6 or more skin lesions that were smaller in size, tending to be bilaterally symmetrical with about 10–40% loss of sensation. The mean age of the patients was 30.4 ± 3.2 years (range 16–50 years) and included 238 men and 48 women. All patients were treated with MDT specific for MB and PB leprosy, as recommended by WHO. The control group consisted of 266 unrelated healthy individuals with mean age of 28.2 ± 4.1 years (range 20–40 years); this group included 222 men and 44 women. None of the controls had any family history of tuberculosis, leprosy or any other related diseases. Both patients and controls belonged to the same geographical area. An informed written consent following the Indian Council of Medical Research (ICMR) norms was obtained from all the individuals whose blood samples were collected. The study was approved by the Jawaharlal Nehru University ethics committee. DNA samples were isolated from peripheral blood using standard phenol–chloroform extraction. In total, 50 ng of genomic DNA was amplified in a 25 µl volume of reaction mixture containing 1× reaction buffer (20 mM Tris pH 8.8, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100), 0.2 mM dNTP, 0.5 µM of each primer and 1.0 U Taq polymerase (New England Biolabs). SNPs were genotyped by direct sequencing of PCR products (BigDye TerminatorCycle Sequencing Kit version 1.1 and ABI PRISM 3100 Avant Genetic Analyzer, Perkin-Elmer Corporation, Foster city, CA, USA). The primers used for SNP T-3575A were: 5-GGTTTTCCTT CATTTGCAGC-3 as the sense primer and 5-ACACT GTGAGCTTCTTGAGG-3 as the antisense primer; for SNP G-2849A and C-2763A, 5-CTGTAATCTCAGCA CTCTGG-3 as the sense primer and 5-GGACTAAA GGGCATGGTGAG-3 as the antisense primer; the SNPs, A-1082T, C-819T and C-592A were amplified by 5-CACAAATCCAAGACAACACT-3 as the sense primer and 5-TATCCTCAAAGTTCCCAAGC-3 as the antisense primer amplifying a region of 681 bp. The A-1082T SNP was genotyped using an internal primer 5-ACAAGGGTACACCAGTGCCA-3 and SNPs C-819T and C-592A were typed using the antisense primer. The amplified PCR products for the other SNPs were sequenced using the reverse primer and the results confirmed by sequencing with the forward primer. Statistical analyses of the polymorphic variant frequencies were performed in a stepwise manner. First, an overall genotype frequencies of patients with leprosy and control subjects were compared using a 3×2 χ^2 test; once a significant overall difference between patients and control subjects was detected ($P < 0.05$), the individual genotypes were analyzed using the unconditional logistic regression model with correction for sex. The genotype frequencies for all the studied SNPs were subjected to Hardy Weinberg Equilibrium analysis. The haplotype frequencies were estimated using haplo.em function in Haplo.Stats software (version 1.2.0) (Schaid et al. 2002) whose progressive insertion algorithm progressively inserts batches of loci into haplotypes of growing lengths, runs the EM steps, trims off pairs of haplotypes per subject when the posterior probability of the pair is below a specified threshold, and then continues these insertion, EM, and trimming steps until all loci are inserted into the haplotype. Partition ligation expectation maximization (PLEM) software was used for constructing genotypes of the haplotypes defined by proximal IL-10 promoter SNPs; which were further analyzed for associations by logistic regression analysis. The patients and control samples were also analyzed independently with two genomic control markers (whose mean heterozygosity was 48%) not known to be associated with leprosy so as to minimize the risk of population stratification bias. The power calculations done using PAWE software (Gordon et al. 2002) indicated that to detect an Odds ratio of 2.5, a sample size of 286 cases and 266 controls would have more than 80% power to detect associations, when the relevant allele had the frequency of more than 0.1%. The statistical software package SPSS version 10.0 (SPSS, Chicago III, IL, USA) was used for logistic regression analysis.

Results and discussion

Table 1 shows the allele and genotype frequency comparison of the six IL-10 promoter SNPs between leprosy patients and healthy controls. The genotype frequencies for these SNPs were found not to deviate significantly from Hardy Weinberg equilibrium in patients nor controls. The allelic and genotypic frequencies of T-3575A, G-2849A, and C-2763A SNPs did not show any significant difference between cases and controls. The −819C, −592C alleles and their C/C genotypes showed a

Table 1 Allele and genotype frequencies of IL-10 promoter SNPs at positions −3575, −2849, −2763, −1082, −819 and −592 in leprosy patients and healthy controls

SNP	Leprosy ($n=282$)	MB ($n=140$)	PB ($n=142$)	Controls ($n=266$)	*P*-value of χ^2 test for overall genotypic frequency[a,b,c]	*P*-value of χ^2 test for individual genotypic frequency[d,e,f]	OR (95%CI)[g,h,i]
-3575							
TT	152 (53.9%)	81 (57.9%)	71 (50.0%)	149 (56.0%)	0.28, 0.31, 0.32		
AT	119 (42.2%)	55 (39.3%)	64 (45.1%)	100 (37.6%)			
AA	11 (3.9%)	4 (2.9%)	7 (4.9%)	17 (6.4%)			
-2849							
GG	229 (81.2%)	110 (78.6)	119 (83.8%)	207 (77.8%)	0.17, 0.14, 0.33		
GA	51 (18.1%)	30 (21.4%)	21 (14.8%)	52 (19.5%)			
AA	2 (0.7%)	0	2 (1.4%)	7 (2.6%)			
-2763							
CC	153 (54.2%)	82 (58.6%)	71 (50%)	152 (57.1%)	0.31, 0.37, 0.26		
CA	118 (41.8%)	54 (38.6%)	64 (45.1%)	98 (36.8%)			
AA	11 (3.9%)	4 (2.9%)	7 (4.9%)	16 (6.0%)			
-1082							
GG	13 (4.6%)	7 (5.0%)	6 (4.2%)	18 (6.8%)	0.26, 0.72, 0.16		
GA	128 (45.4%)	59 (42.1%)	69 (48.6%)	105 (39.5%)			
AA	141 (50%)	74 (52.7%)	67 (47.2%)	143 (53.8%)			
-819							
TT	61 (21.6%)	32 (22.9%)	29 (20.4%)	27 (10.2%)	0.0002, 0.0002, 0.01		Reference
CT	144 (51.1%)	76 (54.3%)	68 (47.9%)	136 (51.1%)		0.004, 0.012, 0.012	2.17 (1.28–3.57), 2.17 (1.19–3.84), 1.78 (1.19–4.00)
CC	77 (27.3%)	32 (22.9%)	45 (31.7%)	103 (38.7%)		0.000, 0.000, 0.005	3.03 (1.78–5.26), 3.84 (2.00–7.69), 2.50 (1.31–4.76)
TT vs CT + CC						0.000, 0.001, 0.005	2.50 (1.49–4.00), 2.63 (1.51–4.76), 2.32 (1.29–4.16)
CC vs CT + TT						0.005, 0.001, 0.163	0.59 (0.41–0.85), 0.46 (0.29–0.74), 0.74 (0.47–1.13)
-592							
AA	60 (21.3%)	33 (23.6%)	29 (20.4%)	27 (10.2%)	0.0004, 0.0001, 0.01		Reference
CA	144 (51.1%)	76 (54.3%)	68 (47.9%)	136 (51.1%)		0.004, 0.016, 0.012	2.94 (1.73–5.26), 3.57 (1.88–7.14), 2.50 (1.31–4.76)
CC	78 (27.7%)	31 (21.1%)	45 (31.7%)	103 (38.7%)		0, 0.001, 0.005	2.94 (0.19–0.58), 3.57 (1.88–7.14), 2.50 (1.31–4.76)
AA vs CA + CC						0.000, 0.001, 0.005	2.43 (1.47–4.00), 2.56 (1.44–4.54), 2.32 (1.29–4.16)
CC vs CA + AA						0.006, 0.002, 0.16	0.60 (0.42–0.86), 0.48 (0.30–2.77), 2.74 (0.47–1.13)

[a] *P*-value for 3×2 χ^2 test of comparison of overall genotype frequencies between total leprosy patients and controls
[b] *P*-value for 3×2 χ^2 test of comparison of overall genotype frequencies between multibacillary (MB) patients and controls
[c] *P*-value of 3× χ^2 test of comparison of overall genotype frequencies between paucibacillary (PB) patients and controls
[d,g] *P*-value and the corresponding OR for comparison of frequencies between total leprosy patients and controls by logistic regression
[e,h] *P*-value and the corresponding OR for comparison of frequencies between multibacillary (MB) patients and controls by logistic regression
[f,i] *P*-value and the corresponding OR for comparison of frequencies between paucibacillary (PB) patients and controls by logistic regression

Table 2 Score tests using a binomial trait (controls vs cases) and ordinal trait (controls vs PB vs MB) to test haplotype association in leprosy

SNP						Haplotype frequencies								
-3575	-2849	-2763	-1082	-819	-592	Controls	PB	MB	Sim. *P*-value[a]	Regression coefficients[a]	OR[a]	95% CI	Sim. *P*-value[b]	Hap-score[b]
T	G	C	A	C	C	0.34	0.27	0.25	0.01	−0.53	0.58	0.37–0.89	0.004	−2.87
T	G	G	G	C	C	0.04	0.02	0.05	0.22	−0.43	0.64	0.32–1.30	0.85	0.18
A	G	A	G	C	C	0.1	0.17	0.09			Baseline		0.57	0.55
T	G	C	A	T	A	0.34	0.42	0.47	0.96	−0.009	0.99	0.64–1.52	0.0002	3.71
A	A	A	G	C	C	0.09	0.08	0.1	0.3	−0.28	0.75	0.44–1.29	0.57	0.3
Rare haplotypes						0.09	0.04	0.04	0.002	−0.94	0.38	0.20–0.72		

Haplotype score tests were calculated using haplo.glm function in Haplo.Stats software (version 1.2.0) which computes the regression of a trait on haplotypes, and possibly other covariates and their interactions with haplotypes. The basis of the algorithm is a two-step iteration process; the posterior probabilities of pairs of haplotypes per subject are used as weights to update the regression coefficients, and the regression coefficients are used to update the haplotype posterior probabilities. The rare haplotypes, those with haplotype frequencies of less than 0.01% (threshold set in our analysis) are pooled into a single category. The ORs for each haplotype was calculated by converting the regression coefficients as per software developer instructions. All the algorithms used and developed for this analysis were in the *R* environment (version 2.1.0).*P*-values were corrected for sex, *Sim. P-value* simulated *P*-value

[a]Binary trait analysis

[b]Ordinal trait analysis

significantly high frequency among controls when compared to total leprosy, MB, and PB patients. Similarly, the frequency of −819T allele and T/T genotype and −592A allele and AA genotype was significantly increased in leprosy patients when compared with healthy controls. The frequency of −819 T/T which was found to be increased in Brazilian leprosy patients (Santos et al. 2002), however, did not show a significant difference between patients and controls in Brazilian population. This probably suggests a differential involvement of these proximal SNPs in susceptibility to leprosy in the two unrelated population groups. The linkage disequilibrium (LD) analysis using EMLD software (http://linkage.rockefeller.edu)] showed a stronger LD among proximal [average $D' = 0.97$] and distal [average $D' = 0.95$] SNPs than in-between proximal and distal SNPs [average $D' = 0.80$] in cases and controls.

Haplotype score tests (Schaid et al. 2002) were performed for three proximal and three distal SNPs independently between cases and controls to find out the presence of a predominant haplotype associated with the disease, if any. The overall differences for the haplotype frequencies for distal SNPs −3575, −2849, −2763, and the proximal SNPs −1082, −819, −592 were significantly different between cases and controls ($P_{\text{corrected for sex}} = 0.002$ and $P_{\text{corrected for sex}} = 0.0004$, respectively). The study of extended haplotypes comprising of all the six SNPs together also showed significant overall difference for haplotype frequencies between cases and controls ($P_{\text{corrected for sex}} = 0.0001$). The contribution of the specific haplotypes in susceptibility to leprosy per se and its severity was further analyzed in this extended haplotype arrangement, using posterior generalized models (GLM) for binomial (cases vs controls) trait. The extended haplotype, −3575T, −2849G, −2763C, −1082A, −819C, −592C was found to be significantly higher in controls than in leprosy patients ($P = 0.01$, OR = 0.58, CI = 0.37–0.89) (Table 2). The ordinal trait analysis confirmed that the frequency for the extended haplotype, −3575T, −2849G, −2763C, −1082A, −819C, −592C, decreased from controls > PB > MB ($P = 0.004$) (Table 2). The ordinal trait analysis also showed that the extended haplotype −3575T, −2849G, −2763C, −1082A, −819T, −592A was significantly associated with the risk of severity of leprosy ($P = 0.0002$) and not to susceptibility to leprosy per se (Table 2). However, the strength of asso-

Table 3 Association with leprosy of genotypes defined by the three proximal promoter IL10 SNPs −1082/-819/-592 haplotypes

IL-10 −1089/-819/-592 genotypes	Leprosy	MB	PB	Controls	*P*-value[a,b,c]	OR (95% CI)[d,e,f]
GCC/ATA	85 (30.1%)	41 (29.3%)	44 (31.0%)	70 (26.3%)		Reference (1.00)
GCC/GCC	12 (4.3%)	6 (4.3%)	6 (4.2%)	18 (6.8%)	0.11, 0.26, 0.17	1.90 (0.85–4.23), 1.78 (0.65–4.89), 2.06 (0.74–5.73)
ACC/ACC	22 (7.8%)	8 (5.7%)	14 (9.9%)	51 (19.2%)	0.001, 0.002, 0.02	2.86 (1.58–5.17), 3.88 (1.66–9.05), 2.29 (1.13–4.63)
ATA/ATA	61 (21.7%)	32 (22.8%)	29 (20.4%)	27 (10.2%)	0.03, 0.04, 0.10	0.55 (0.31–0.95), 0.51 (0.26–0.97), 0.58 (0.31–1.22)
GCC/ACC	43 (15.2%)	18 (12.9%)	25 (17.6%)	34 (12.8%)	0.88, 0.77, 0.64	0.95 (0.55–1.66), 1.10 (0.55–2.20), 0.85 (0.45–1.62)
ACC/ATA	59 (20.9%)	35 (25.0%)	24 (16.9%)	66 (24.8%)	0.20, 0.70, 0.07	1.36 (0.85–2.17), 1.11 (0.63–1.96), 1.72 (0.94–3.15)

[a,d]*P*-value and the corresponding OR for comparison of frequencies between total leprosy patients and controls by logistic regression

[b,e]*P*-value and the corresponding OR for comparison of frequencies between multibacillary (MB) patients and controls by logistic regression

[c,f]*P*-value and the corresponding OR for comparison of frequencies between paucibacillary (PB) patients and controls by logistic regression

ciation (OR) for the haplotypes could not be measured in ordinal trait analysis because of non availability of ordinal trait analysis in haplo.glm function of Haplo.Stats software (version 1.2.0).

The results of association of individual promoter SNPs suggests that the effect of proximal promoter SNPs (−1082A > G, −819C > T, −592C > A) in determining risk/protection to leprosy is more pronounced than distal SNPs (−3575T > A, −2849G > A, −2763C > G). The genotypes defined by the proximal promoter SNP(−1082, −819, −592) haplotypes also showed significant preponderance of ACC/ACC haplotype in controls and ATA/ATA haplotype in patients (Table 3). The analysis of the entire region encompassing −4 to 0.5 kb, using genotyping data of the six IL-10 promoter SNPs suggests that the extended haplotype, −3575T, −2849G, −2763C, −1082A, −819C, −592C is protective while −3575T, −2849G, −2763C, −1082A, −819T, −592A is a susceptible haplotype. Our results do not as yet provide a clear answer as to why −3575T, −2849G, −2763C, −1082A, −819C, −592C is protective and −3575T, −2849G, −2763C, −1082A, −819T, −592A is a susceptible haplotype. The proximal promoter polymorphisms have been reported to define 'high' (−1082G/-819C/-592C), 'medium' (−1082A/-819C/-592C) and 'low' (−1082A/-819T/-592A) expressing genotypes for IL-10 (Turner et al. 1997). The mosaics of distal and proximal promoter elements reflecting high and low responses are, however, still unknown. It will be interesting to find out how the extended haplotype influences the expression of IL-10 in in-vivo conditions. We did not investigate the effect of IL-10 polymorphisms on cytokine production in patients and control subjects. Therefore, it is still important to analyze whether the genotypes that appear to be associated with increased or decreased levels of IL-10 could be used to select patients and controls in our population in whom these increased or decreased levels are observed.

Although the frequency of −819T/T was found to be increased in Brazilian leprosy patients (Moraes et al. 2004), it did not show significant differences between patients and controls suggesting differential involvement of these proximal SNPs in susceptibility to leprosy. The frequency differences in the studied Indian population and that of Brazil for IL-10 promoter SNP genotypes and haplotypes probably explains the involvement of different genotypes and haplotypes in the resistance/susceptibility to leprosy or its types. The patterns of LD across the relevant chromosomal region may differ between populations and contribute to heterogeneity among associations. The strength of LD among the proximal and distal promoter SNPs was comparable to the strength of LD observed for same markers for Brazilian population. These observations highlight the differences in relative importance of these SNPs as susceptibility loci in disease manifestation in the Indian population and the population studied previously. The present study showed that the haplotypes, −3575A, −2849G, −2763C, and −3575T, −2849A, −2763C, defined as a marker of resistance and susceptibility to leprosy, respectively; in Brazilian population (Moraes et al. 2004) were observed in a very low frequency both in controls and patients in the studied Indian population group. The rare haplotypes when pooled together, showed an overall significant effect, suggesting that any effect of a specific haplotype in disease resistance and severity cannot be ruled out, however, the overall contribution to disease resistance at the population level for these rare haplotype would be limited. The haplotype −3575T, −2849G, −2763C, −1082A, −819C, −592C with a relatively high frequency in our study suggested it to be a significant contributor to the disease resistance in our population though with a modest effect (OR = 0.58). These results corroborate with the fact that the outcome of mycobacterial infection involves complex interactions between several other host genes and also highlight the role of IL-10 in early and late phases of leprosy infection.

The involvement of IL-10 polymorphisms in the outcome of leprosy in two ethnically distinct populations, Brazilian and Indian, suggests that IL-10 region needs to be further investigated both in genetic and functional studies.

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