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ARTICLE

Association study of major risk single nucleotide polymorphisms in the common regulatory region of PARK2 and PACRG genes with leprosy in an Indian population

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Single nucleotide polymorphisms (SNPs) in the regulatory region shared by PARK2 and PACRG have been identified as major risk factors for leprosy susceptibility in two ethnically distinct populations. We investigated the association of six SNPs present in this regulatory region with leprosy susceptibility in an Indian population. Genotyping was performed by direct PCR sequencing in 286 leprosy patients and 350 healthy controls. Our results showed that T allele of SNPs PARK2_e01 (−2599) and 28 kb target_2_1 was significantly associated with susceptibility to leprosy *per se* ($P = 0.03$ and 0.03, respectively). The T allele of SNPs PARK2_e01 (−2599) showed a significant recessive effect ($P = 0.04$) in susceptibility to leprosy in Indian population as against the dominant effect of haplotype T-C of the major risk SNPs PARK2_e01 (−2599) and rs1040079 in Brazilian and Vietnamese population. However, after bonferroni corrections, these significant differences disappeared. Haplotype analysis also showed a lack of significant association of any haplotype with cases or controls. The noninvolvement of major risk SNPs in the regulatory region of PARK2 and PACRG locus with leprosy susceptibility in Indian population highlights the differential effect of these SNPs in regulating genetic susceptibility to leprosy in different populations.
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Leprosy or Hansen's disease is a chronic and debilitating disease that affects an estimated 700 000 people each year.[1] Leprosy is characterized by a wide spectrum of clinical manifestations that depends upon the host cell mediated immune response against the pathogen.[2] At one pole, tuberculoid leprosy patients manifest a strong cellular immune response that results in a few localized, often self healing paucibacillary lesions. At the other end, lepromatous leprosy patients exhibit poor cell mediated immunity against *Mycobacterium leprae* antigens leading to a disseminated disease involving extended multibacillary lesions of skin and nerves. Evidence from segregation and twin studies suggest the existence of a strong genetic component for susceptibility to leprosy in human populations.[3,4] Numerous case control studies have identified variants in VDR, HLADR2 specificities, TAP1 and TAP2, CTLA4, COL3A,

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SLC11A1 (also called NRAMP1) and TNF-α to be associated with leprosy or its subtypes.[5] These associations reflect differential susceptibility to this polygenic disease in different populations. Genome wide linkage scans so far have led to the identification of a chromosomal region 10p13, for loci controlling susceptibility to the PB form of leprosy[6] and a chromosomal region 6q25–26 harboring variants in the common regulatory region of PARK2 and PACRG genes as risk factors for susceptibility to leprosy *per se*.[7] PARK2, a ubiquitination E3 ligase involved in delivery of polyubiquitinated proteins to the proteasomal complex, as yet has an undefined role in leprosy pathogenesis. The function of PACRG in leprosy pathogenesis is unknown but also has been linked to the ubiquitin–proteasome system.[8,9]

India carries the majority of the global burden of leprosy. It was, therefore, pertinent to investigate whether the SNPs, implicated previously as major risk alleles, were also associated with susceptibility to leprosy in an Indian population group.

In order to identify the possible association of PARK2 and PACRG regulatory region SNPs with susceptibility to leprosy *per se*, or with different clinical forms of the disease, we compared leprosy patients to controls, and also compared PB and MB leprosy patients with controls. Power calculations carried out using PS software (www.biostat.mc.vanderbilit.edu/twiki/bin/view) showed that our sample size of 286 patients and 350 controls had more than 75% ($P<0.05$) power to detect an OR of 2.0 when the relevant allele has frequency >0.10. Table 1 depicts the genotype distribution and allele frequency observed for the SNPs studied. Since the minor allele frequency of the SNP rs1893450 was <0.05, it was not further analyzed for association scan. Genotype frequencies were found not to deviate significantly from Hardy–Weinberg equilibrium

Table 1 Allele and genotype frequencies of the SNPs, 10 kb_target_5_2, PARK2_e01 (−697), PARK2e_01 (−2599), 28 kb target_2_1, rs1040079 and rs1893450 in multibacillary and paucibacillary leprosy patients and healthy controls

SNP (denomination)	*Leprosy (n = 286)*	*MB (n = 144)*	*PB (n = 142)*	*Controls (n = 350)*	*P-value*[a,b,c]	*P corrected**
10 kb_target_5_2 (A)						
CC	38 (13.3%)	17 (11.8%)	21 (14.8%)	33 (9.4%)	Reference	
CT	129 (45.1%)	66 (45.8%)	63 (44.4%)	169 (48.3%)	0.12, 0.40, 0.09	
TT	119 (41.6%)	61 (42.4%)	58 (40.8%)	148 (42.3%)	0.18, 0.52, 0.12	
CC *vs* CT+TT					0.12, 0.42, 0.08	
TT *vs* CT+CC					0.88, 0.95, 0.77	
T	66%	65%	63%	64%	0.39, 0.72, 0.30	
PARK2_e01(-697) (B)						
CC	21 (7.3%)	10 (6.9%)	11 (7.7%)	28 (8.0%)	Reference	
CT	105 (36.7%)	58 (40.3%)	47 (33.1%)	132 (37.7%)	0.84, 0.60, 0.82	
TT	160 (55.9%)	76 (52.8%)	84 (59.2%)	190 (54.3%)	0.72, 0.77, 0.77	
CC *vs* CT+TT					0.57, 0.69, 0.92	
TT *vs* CT+CC					0.68, 0.77, 0.32	
T	74%	73%	76%	73%	0.64, 0.94, 0.40	
PARK2_e01(-2599) (C)						
CC	5 (1.7%)	2 (1.4%)	3 (2.1%)	11 (3.1%)	Reference	
CT	62 (21.7%)	31 (21.5%)	31 (21.8%)	97 (27.7%)	0.54, 0.45, 0.80	
TT	219 (76.6%)	111(77.1%)	108 (76.1%)	242 (69.1%)	0.20, 0.23, 0.45	
CC *vs* CT+TT					0.27, 0.28, 0.53	
TT *vs* CT+CC					**0.04**, 0.08, 0.12	**0.6**
T	87%	88%	87%	83%	**0.03**, 0.05, 0.12	**0.45**
28 kb_target_2_1 (D)						
CC	5 (1.8%)	2 (1.5%)	3 (2.1%)	14 (4.0%)	Reference	
CT	67 (23.4%)	32 (22.2%)	32 (22.57%)	96 (27.4%)	0.24, 0.26, 0.50	
TT	214 (74.8%)	107(74.3%)	107 (75.4%)	240 (68.6%)	0.08, 0.13, 0.25	
CC *vs* CT+TT					0.11, 0.16, 0.30	
TT *vs* CT+CC					0.07, 0.18, 0.13	
T	87%	87%	87%	82%	**0.03,** 0.07, 0.09	**0.45**
rs 1040079 (E)						
CC	12 (4.2%)	5 (3.5%)	7 (4.9%)	24 (6.9%)	Reference	
CT	110 (38.5%)	55 (38.2%)	55 (38.7%)	128 (36.6%)	0.15, 0.16, 0.39	
TT	164 (57.3%)	84 (58.3%)	80 (56.3%)	198 (56.6%)	0.15, 0.15, 0.45	
CC *vs* CT+TT					0.14, 0.15, 0.42	
TT *vs* CT+CC					0.82, 0.68, 0.96	
T	77%	77%	76%	75%	0.47, 0.39, 0.78	

Table 1 (*Continued*)

SNP (denomination)	*Leprosy (n = 286)*	*MB (n = 144)*	*PB (n = 142)*	*Controls (n = 350)*	*P-value*[a,b,c]	*P corrected**
rs 1893450						
TT	265 (92.7%)	132(91.7%)	133 (93.7%)	324 (92.6%)		
CT	21 (7.3%)	12 (8.3%)	9 (6.3%)	25 (7.1%)		
CC	0	0	0	1 (3.5%)		
T	96%	96%	96%	96%		

[a]*P*-value for comparison of frequencies between total leprosy patients and controls.
[b]*P*-value for comparison of frequencies between multibacillary (MB) patients and controls.
[c]*P*-value for comparison of frequencies between paucibacillary (PB) patients and controls.
P corrected * are the corrected *P*-values after bonferroni correction for the SNPs that showed significant association.
Bonferroni correction of 15 (five SNPs tested in three patient groups) was applied for multiple testing.

A total of 286 leprosy cases were recruited on presentation to the Hansen's outpatient unit of Lok Nayak Jai Prakash Hospital (New Delhi, India). Each patient was examined by an experienced physician who assessed the type of leprosy on the basis of the appearance and distribution of skin lesions, the nature of anesthesia, slit-skin smear examinations, and the presence of thickened peripheral nerves. Following slit-skin smear tests at three or more sites (including ear lobe, nostril, and skin lesions), the clinical diagnosis was reassessed by a panel of two clinical leprologists. The classification of the patients was based on clinical and histological criteria (Ridley & Jopling, 1966). Patients were classified as multibacillary (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 paucibacillary (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 presence of large well-defined skin lesions, which were <5 in number, dry and with almost 90–100% loss of sensation. Multibacillary leprosy was characterized by presence of six 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 350 unrelated healthy individuals with mean age of 28.2 ± 4.1 years (range 20–40 years); this group included 297 men and 53 women. Both the patient and control groups consisted of individuals belonging to the Hindu, Muslim, or Christian religions. Among cases of multibacillary leprosy, 80% were Hindus (45% Shudras, 30% Kaisthas, 5% Brahmins), 17% were Muslims, and 3% others. The respective proportions were 77% (40, 30, 7%), 14, and 3% for those with paucibacillary leprosy, and 78% (40, 29, 9%), 17, and 3% for controls. None of the controls had any family history of tuberculosis, leprosy or any other related diseases. Both patients and controls were selected from the same geographical area of leprosy prevalence in Delhi area. To investigate the extent of admixture and homogeneity testing, we analyzed the two genomic control markers in both cases and controls independently by distance *Fst* values between the different population groups in both the data sets using ARLEQUIN 2000 software.[10] 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 $\times$ 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). Single nucleotide polymorphisms 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 PARK2_e01 (−2599) were: 5-AGCATT GGAATGTTGACTTATC-3 as the forward primer and 5-TCAATTTCATAACCAATATTTACAT-3 as the reverse primer; for SNP rs1040079, 5-GGACT AAAGGGCATGGTGAG-3 as the forward primer and 5-GGACTAAAGGGCATGGTGAG-3 as the reverse primer; for SNP 10 kb_target_5_2, 5-ATTCTC TTCTCTCCCCTCCA-3 as the forward primer and 5-ACCTCTTACCCTCTTTTTAGTGTG-3 as the reverse primer; for SNP PARK2_e01 (−697), 5-GTGCA CCAGCCTCACTCTCT-3 as the forward primer and 5-GCGGTCCAGAAACCATAAGT-3 as the reverse primer; for SNP 28 kb target_2_1, 5-CTTGT TTTGGCCCGTAAGTATG-3 as the forward primer and 5-AGAACCAACGCCACAATCAC-3 as the reverse primer; for SNP rs1893450, 5-AGATCC TCAATATATGCCCATG-3 as the forward primer and 5-AGGGATAAAGATGCAAAAGTCA-3 as the reverse primer. The amplified PCR products were sequenced using the reverse primer and the results confirmed by sequencing with the forward primer. The statistical significance of differences in allele and genotype frequencies of the SNPs in patients *vs* controls was analyzed using the unconditional logistic regression model with correction for sex. The magnitude of LD was evaluated in D' and r^2 using the haplotype frequencies estimated by the EMLD program (http://linkage.rockefeller.edu). The statistical software package SPSS version 10.0 (SPSS, Chicago III, Illinois, USA) was used for all statistical analysis. Bonferroni correction of 15 (five SNPs tested in three patient groups) was applied for multiple testing.

for controls nor patients. The T allele of SNPs PARK2_e01 (−2599) and 28 kb target_2_1 was found to be significantly associated with leprosy. The T allele of SNP PARK2_e01 (−2599) showed a significant recessive effect with susceptibility to leprosy *per se*. However, these significant associations did not sustain after bonferroni corrections. Besides, allele and genotype frequencies of the remaining SNPs were not found to be significantly different between total leprosy patients and controls and between patients with MB or PB form of leprosy and healthy controls. The linkage disequilibrium pattern (D' and r^2 values) between the five SNPs (10 kb_target_5_2, PARK2_e01 (−697), PARK2_e01 (−2599), 28 kb target_2_1, rs1040079, referred here as A, B, C, D, E, respectively, showed strong linkage disequilibrium between markers B, C, D, E with average D' value of 0.90 between them. (Table 4). However, the marker A (10 kb_target_5_2) showed weak linkage disequilibrium with markers B, C, D, E with average D' value of 0.67 between them.

Haplotypes derived of a combination of three, four and five SNPs were analyzed to assess the frequency differences between cases and controls (Table 2). The haplotype frequencies were estimated using haplo.em function in Haplo.Stats software (version 1.2.0)[11] 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. None of the haplotype combinations showed a significant association. Although the global haplotype score tests for BCDE haplotype combinations showed close to significant association, this region when further analyzed to investigate the effect of any specific haplotype in association with disease susceptibility (Table 3) showed no significant association for any specific haplotype (Table 4).

These five single nucleotide polymorphisms in the common regulatory region of PARK2 and PACRG genes have been identified as major risk factors for leprosy in two ethnically distinct populations.[7] Among them, PARK2_e01 (−2599) and rs1040079 were the two most significantly associated SNPs. Independently these SNPs conferred relatively lower risk in a recessive manner whereas these SNPs in *cis* as a haplotype showed significant dominant effect in susceptibility to leprosy *per se*.[7] The risk allele T of SNP 28 kb target_2_1 showed differential associations with susceptibility to leprosy between Brazilian and Vietnamese population.[7] It was, however, interesting to find in our study that the susceptibility to leprosy *per se* is not confined to the 80 kb block region of PARK2 and PACRG locus in Indian population. There was a significant difference in the prevalence of the allele T of PARK2_e01 (−2599) in Indian population and the two populations (Brazilian and Vietnamese). We did not find a significant association with the risk SNPs or haplotypes with leprosy *per se* or with different clinical forms of leprosy in an Indian population, suggesting heterogeneity in association

Table 2 Scan of the PARK2 and PACRG regulatory region SNPs combining genotypes in different haplotypes

	Corrected for sex	
Haplotype combination	*P*-value	*Simulated P-value*
Three loci haplotype		
A/B/C	0.295	0.303
B/C/D	0.228	0.236
C/D/E	0.160	0.156
Four loci haplotype		
A/B/C/D	0.483	0.490
B/C/D/E	0.075	0.065
Five loci haplotype		
A/B/C/D/E	0.330	0.325

A = 10 kb_target_5_2, B = PARK2_e01 (−697), C = PARK2_e01 (−2599), D = 28 kb target_2_1, E = rs1040079.
Test of association for the scan (overall differences between cases and controls) were performed as described by Schaid *et al*.[11]

Table 4 The linkage disequillibrium pattern between the four SNPs in common regulatory region of PARK2 and PACRG

		D′		*r*^{2}	
SNP PAIR		*Case*	*Control*	*Case*	*Control*
A	B	0.73	0.76	0.1	0.1
A	C	0.73	0.63	0.04	0.02
A	D	0.55	0.55	0.02	0.03
A	E	0.67	0.68	0.08	0.08
B	C	0.99	0.99	0.05	0.07
B	D	0.8	0.99	0.04	0.07
B	E	0.84	0.9	0.61	0.75
C	D	0.97	0.9	0.84	0.8
C	E	0.83	0.92	0.03	0.05
D	E	0.75	0.93	0.03	0.06

A = 10kb_target_5_2; B = PARK2_e01 (−697); C = PARK2_e01 (−2599); D = 28 kb target_2_1; E = rs1040079.

Table 3 Score tests using a binomial trait (controls *vs* cases) to test haplotype association in leprosy

SNP			*Haplotype frequencies*			
B/C/D/E	*Control*	*Patients*	*Regression Coefficients*	*Sim. P-value*	*OR*	*95% CI*
T/T/T/C	0.011	0.024	0.73	0.14	2.09	0.78–5.6
T/T/C/T	0.018	0.011	−0.32	0.45	0.71	0.30–1.69
T/C/C/T	0.153	0.120	−0.31	0.06	0.72	0.51–1.02
C/T/T/T	0.034	0.052	0.35	0.20	1.43	0.81–2.50
C/T/T/C	0.233	0.203	−0.23	0.10	0.79	0.59–1.05
Rare haplotypes	0.020	0.014	−0.71	0.21	0.48	0.15–1.5
T/T/T/T	0.531	0.576			1.00	
Reference haplotype						

P-values were corrected for sex; Sim. *P*-value = simulated *P*-value.
Haplotype score tests were calculated using haplo.glm function in Haplo.Stats software (version 1.2.0)[11] 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).

of these SNPs with susceptibility to leprosy in different populations. The association of nonfunctional variants depends upon the patterns of LD across the relevant chromosomal region, which may differ between populations and contribute to heterogeneity among associations. The strength of LD among the four markers (B, C, D, E) studied in our population was comparable to the strength of LD observed for same markers for Brazilian and Vietnamese population. These observations highlight the differences in relative importance of these SNPs as susceptibility markers in disease manifestation in the Indian population and the populations studied previously. A number of association studies in the past have also suggested the prevalence of differential genetic susceptibility between populations.[5] This is supported by genome wide linkage scans of several complex diseases, such as type 2 diabetes, where both different and overlapping chromosome regions were linked in different populations. It has been shown that such differential susceptibility could extend between different caste groups within a population. The genetic heterogeneity in linkage of chromosomal region 20p12 with the susceptibility to PB form of leprosy between two population groups of South India corroborates the existence of genetic diversity between caste groups in India.[12] The risk of population stratification bias due to differences in the ethnic background between patients and controls and variations of allele frequencies according to ethnic background was minimized by including patients and controls matched for the same ethnic background, residing in the same geographical area of leprosy prevalence and by admixture testing using two genomic control markers. We did not observe a significant difference in *Fst* distance values in-between different religious groups within cases (average $Fst = 0.0007$) and controls (average $Fst = 0.0008$) suggesting that the studied cases and controls belong to a homogenous population group. Further, the samples when analyzed independently with same genomic control markers, (mean heterozygosity of 48%), did not show any association with cases and controls.

The noninvolvement of SNPs in the common regulatory region of PARK2 and PACRG locus with leprosy in Indian population shows that the effect of the SNPs in this region in regulating genetic susceptibility to leprosy appears to be differential in Indian population when compared to Brazilian and Vietnamese populations. It will be interesting to investigate whether the spectrum of variations within other regions of PARK2 and PACRG loci, apart from the presence of a global risk SNP PARK2_e01 (−2599), are also involved in disease susceptibility in Indian population. Also, it will be worthwhile to examine the role of other modifier gene(s) in the background of risk alleles in PARK2 and PACRG locus in providing susceptibility to leprosy.

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