PARK2 and pro-/anti- inflammatory cytokine gene interactions contribute to the susceptibility to Leprosy

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Title: PARK2 and pro-/anti- inflammatory cytokine gene interactions contribute to the susceptibility to Leprosy

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Abstract

Objectives: Cytokines and related molecules in immune-response pathways seem important in deciding the outcome of the host-pathogen interactions towards different polar forms in Leprosy. Here we study the role of significantly associated and functionally important SNPs in these genes, published independently from our research group, through combined interaction with an additional analysis of the *in-silico* network outcome, to understand how these impact the susceptibility towards the disease, leprosy.

Design: The study was designed to assess an overall combined contribution of significantly associated individual SNPs to reflect on epistatic interactions and their outcome in the form of the disease, leprosy. Further *in-silico* approach was adopted to carry out Protein-Protein interaction study between PARK2 and pro-/anti- inflammatory cytokines.

Setting: Population based case-control study involved the data of North India. Protein-Protein interaction networks were constructed using Cytoscape.

Participants: Study included 2305 Northern Indians samples (829 Leprosy Patients; 1476 Healthy controls).

Primary and secondary outcome measures: For genotype interaction analysis, all possible genotype combinations between selected SNPs were used as an independent variable in a model using logistic regression with the forward likelihood ratio method, keeping the gender as a covariate.

Results: Interaction analysis between PARK2 with significant SNPs of anti-/pro- inflammatory cytokine genes and the genes spanning the HLA region (6p21.3) i.e., BAT1 to BTNL2-DR in a case-control comparison, showed that combined interaction analysis of PARK2, TNF, BTNL2-DR, IL10, IL-6 & TGFBR2 increased the risk towards leprosy (OR=2.54) and PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B & IL10RB provided increased protection (OR=0.26) in comparison to their individual contribution.

Conclusions: The epistatic SNP-SNP interaction involving the PARK2 and cytokine genes provide an additive risk towards leprosy susceptibility. Further, *in-silico* Protein-Protein Interaction (PPI) of PARK2 and important pro-/anti inflammatory molecules indicate that PARK2 is central to immune regulation, regulating the production of different cytokines upon infection.

Keywords: PARK2, pro-/anti- inflammatory cytokine genes, Leprosy, Gene interaction

Article Summary

Strengths and limitation of this study

- Many of the genetic studies lack replication in different population groups, explaining the heterogeneity in associated genes and genomic regions. This may be due to the complex nature of a disease. The complexity, however, could partly be delineated at genetic level by assessing the quantum of the contribution of different loci to the disease in a combined manner instead of their individual role.
- Our study highlights the importance of a combined effect of the important cytokine and other immune regulatory genes, whose combined effect in diverse genotype

combinations, provide either increased risk or protection towards the complex genetic disease, Leprosy.

- Genetic interaction and an additional in-silico pathway analysis provided an overall perspective on how PARK2 gene product; parkin, acts as a centrally placed molecule, playing an important role in regulating different pathways of the immune response and susceptibility to Leprosy. This conclusion needs further support with future experiments in vitro or in vivo of T cell responses in different genetic backgrounds of the identified networks in this study.

Introduction

Leprosy caused by *Mycobacterium leprae* is a chronic infectious disease, characterized by clinically defined polar forms in which pathology and immunology are inextricably related, providing a critical model to explore the immuno-regulatory mechanisms in humans. At one pole, tuberculoid form is associated with a strong cell mediated immunity and T helper 1 (Th1) cytokine profile and at the other end of the spectrum, the lepromatous form is associated with a strong humoral response and T helper 2 (Th2) cytokine profile. Cytokines and other related molecules of the immunological pathways thus seem to be a part of significant group of candidates that are apparently critical for the host-pathogen interactions, where the outcome of the disease is majorly dependent on the host factors controlling the immune response, especially when *M. leprae* possesses lowest level of genetic diversity [1]. This is supported by various studies of familial clustering [2], twin studies [3], complex segregation analysis [4 5], test of analysis with the HLA genes [6] including recent genome-wide association studies, [7 8] and studies of several genes that modulate cell-mediated immunity, with a role in either susceptibility to leprosy per se or to leprosy types [9]. Various candidate gene studies and genome-wide approaches have implicated polymorphisms in cytokine genes, whose protein products are part of important immune modulatory molecules, playing a major role in influencing host-pathogen interactions and determine the outcome of many infectious and autoimmune diseases [10-16]. However, only a few observations have been replicated unequivocally in different population groups, suggesting the polygenic nature of the disease with a high degree of heterogeneity among different populations.

We, in the recent past, have studied various candidate genes of pro-/anti-inflammatory cytokines in two independent population groups, North and East India-Orissa; and found strong association with IL-10, IL-10RB, TGFBR2, IL-6 [14], IL-12B [17]. Fine-mapping of a specific 6p (HLA) chromosomal region revealed a significant association of important candidates, BAT1, LTA, TNF and BTNL2 [16]. In a subsequent study of the 6q chromosomal region, involving the overlapping regulatory domain of PARK2-PACRG genes, revealed an involvement of significant SNPs and presence of a differential LD structure in Indian populations as compared to Vietnamese [18]. The latter observation and the functional role of PARK2, as a ubiquitin ligase, has recently been shown in providing resistance to intracellular pathogens [19] through ubiquitin mediated autophagy. Further the involvement of parkin in regulating production of cytokines upon infection [20], indeed provides strong hint for any functional variation in the gene having profound effect in modulating the expression of the immune-regulatory genes. The importance of all the studied genes [14-18] in the network of immune-response necessitated the analysis of an interaction between these genes as a whole to understand their contribution together towards the susceptibility of the complex disease, Leprosy; where the outcome of the infection in all probabilities depends on the nature of gene interactions between the genes with the potential of contributing to the immune pathology.

The aim, therefore, of this study was to assess an overall interaction between the significant and functionally important SNPs studied in a case-control comparison of the samples from New Delhi, in Northern India; where most of these SNPs were replicated in an unrelated East Indian-Orissa population. These included for an overall interaction the PARK2 gene significant SNPs [18] with the significant SNPs of anti-inflammatory cytokine genes (IL-10, IL-10RB, TGFBR2, IL-6) [14], pro-inflammatory cytokine genes (TNF-alpha, LT-alpha, IL-12B) and the genes spanning the HLA region of the chromosome 6p21.3 i.e., BAT1 to BTNL2-DR [16 17] to evaluate their combined contribution towards the outcome of the complex infectious disease; Leprosy.

Methods

The study involved the revisit of our published work on individual candidate genes and regions, studied in North Indian population groups in case-control comparison. The data compiled was

from a group of 2305 samples from Northern India (including 829 Leprosy patients and 1476 unrelated healthy control subjects from North India) [14 16-18] with a complete coverage of genes belonging to pro-, anti-inflammatory cytokines, selected HLA regions in 6p21.3 and common regulatory region of PARK2/PACRG genes located at 6q26 region. The patients' group was classified as pauci-bacillary (PB) or multi-bacillary (MB) according to the Ridley and Jopling criteria [21], including 421 Pauci-bacillary patients and 408 Multi-bacillary patients, with a mean age of 32.30±3.2 years (range 6-80 years). All these patients were under treatment with multidrug therapy (MDT) specific for multibacillary (MB) and paucibacillary (PB) leprosy, as recommended by the World Health Organization.

For genotype interaction analysis, all possible genotype combinations between selected SNPs (pairwise or multiple genes) were ascertained from a MassArray platform for the given genotypes of SNPs. These interactions were tested as an independent variable in a model using logistic regression with the forward likelihood ratio method, keeping the gender as a covariate. In this selection method, entry testing based on the significance of the score statistics and removal testing based on the probability of a likelihood ratio statistics was based on the maximum partial likelihood estimates. Furthermore, in multiple gene interaction analysis, all interactions with either risk or protective effect were combined against other interactions to observe an overall effect of the risk Vs protective interactions. These analyses were performed using statistical software package SPSS V.17.0 (SPSS, Chicago III, Illinois, USA) for Windows. *P* Value was considered significant at and below 0.05.

In-silico approach to assess the network of the genes in a Protein-Protein Interaction (PPI) of PARK2, using Agile Protein Interaction Database (APID), a comprehensive resource for protein interaction data, automatically accessed by Cytoscape [22] through the dedicated plugin APID2NET [23], was carried out to understand the involvement of the studied interactome. APID integrates in a single web-based tool all known experimentally validated PPI from BIND [24], BioGRID [25], DIP [26], HPRD [27], IntAct [28] and MINT [29] databases.

Results and Discussion

Leprosy, an ideal model of a chronic human complex infectious disease, provides an opportunity to dissect the components of the host dependent polygenic susceptibility to this disease. In order

to understand the role of multiple genes together, an interaction analysis was carried out between the genotype status of functionally different variants of different genetic loci involved in immune response, with an expected combined effect on the outcome of the disease in different polar forms of the disease. The interaction analysis carried out between PARK2 gene regulatory region SNPs (rs9365492 & rs9355403) [18] and SNPs of the anti-inflammatory cytokines [14] provided a significant risk towards the leprosy susceptibility; combining individually with SNPs of IL-10 (OR=1.99), IL-6 (OR=1.33) and TGFBR2 (OR=1.29) cytokine genes. However, with IL10RB (receptor beta), the result showed a significant protection towards the disease (OR=0.61). Similar analysis between PARK2 SNPs with pro-inflammatory cytokine genes TNF-alpha and BTNL2-DRA interval (showing strong LD with the BTNL2 promoter SNPs) [16] provided a significant risk towards leprosy susceptibility with OR=2.10 and OR=5.40, respectively. However, the SNPs of BAT-1, NFKABIL1, LTA, TNF-LTB, IL12B [16 17] provided a significant protection towards leprosy with OR=0.65, OR=0.58, OR=0.61, OR=0.54 and OR=0.71, respectively (Table 1, Figure S1).

In the second step of combined interaction analysis with all the genes, either providing protection or risk towards leprosy, showed that the combined genotypic interaction analysis of the SNP loci PARK2, TNF, BTNL2-DR, IL10, IL-6 and TGFBR2, further increased the risk of leprosy (OR=2.54); and a similar combined analysis for loci PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB, increased protection towards leprosy (OR=0.26), in comparison to their individual contribution (Table 2 (a, b)).

PARK2, encoding the E3 ubiquitin ligase protein-parkin, has been shown to involve in the cellular ubiquitination metabolism [30], providing resistance to intracellular pathogen via ubiquitin mediated autophagy [19]. Also, respond to infection in a regulated way by producing important cytokines [20], suppressing molecules that limit pro-inflammatory - IL-2 [31], TNF-alpha cytokine production and enhancing the production of anti-inflammatory cytokines, IL-4, IL-10, and IL-13 [32-37]. All these observations indicate the *in-vivo* importance of PARK2 gene product-parkin to be centrally involved in immune-regulatory network, working against the invading mycobacteria; justifying to find out an interesting relation between important immune-regulatory cytokines with PARK2, essentially shown to be involved in the host responses to *M. leprae* [38] and also for pathogenesis of the disease [8 39].

The genotypic analysis carried out in the North Indian population revealed PARK2 to be centrally involved as an important immuno-regulator molecule at the genetic level. This result opens the way for future studies to see the *in-vivo* effect of PARK2 variations at the expression level for all the important pro-/anti- inflammatory cytokine proteins involved in the immune system. To have an *in-silico* understanding of immuno-regulatory protein interactions we further carried out *in-silico* analysis to identify the Protein-Protein Interaction (PPI) of PARK2. We used APID2NET and Cytoscape tools for PARK2 interaction Data retrieval, providing a total of 43 PARK2 interacting proteins. However, the result did not provide any direct interaction of the PARK2 with the cytokines studied by us in North Indian population [14 16-18]. However we considered 43 PARK2 interacting proteins for pathways analysis by using KEGG , BioCarta, Nci-Nature and Reactome tools, resulting these 43 proteins to be involved in 253 different pathways (without removing overlapping pathways). Similarly, in the second step we identified pathways for 11 cytokine proteins studied by us in North Indian population [14 16-18], this results the involvement of 5 cytokine proteins; IL12B, IL6, TNF, TGFR2 & IL10 in 94 pathways, not involving BTNL2, BAT1, NFKBIL, LTA, IL10RB2 & BTNL2-DR in any pathways. Comparing both pathways; 253, PARK2 interacting proteins pathways and 94, cytokine proteins pathways; reveals 27 commonly involved pathways, via CASP8, CUL1, CCNE1 & CCNA proteins, involving only 5 (IL12B, IL6, TNF, TGFR2 & IL10) out of 11 cytokine proteins studied in North Indian population (Figure-1), connecting majorly through Toll-like receptor signaling pathways (Figure 1, Table S1).

The above interaction and pathway analysis allows us to propose that the complex genetic background is the predominant factor for the outcome of the disease, where the combined effect of the variant risk alleles of the PARK2 gene, responsible for affecting transcription binding site and lowering the expression of the reporter gene by *in-vitro* experiment [18], along with the risk alleles of the anti-inflammatory cytokines genes - IL-10, IL-6, TGFBR2, responsible for lowering the CMI response towards the invading bacteria and pro-inflammatory cytokines - TNF-alpha; is responsible in providing highly significant risk towards leprosy. The study opens a way for future *in-vivo* work of immune-response read outs in complex variant genomic backgrounds to understand the wide gap in understanding the balance in the network of all the

immune regulatory molecules operational in providing either susceptibility or resistance towards disease.

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Data sharing statement: Extra data is made available by contacting corresponding author at Email ID: bamezai@hotmail.com.

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Figure 1: PARK2 Interaction Analysis: unfilled circles showing the PARK2 interacting proteins, Light grey circles showing protein links between the PARK2 interacting protein and 5 cytokines protein (dark grey circles) study by us in North India population

Figure S1: a. Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs rs9365492 and rs9355403 with the SNPs of gens providing Risk toward the Leprosy. Genotypes/Alleles in red color represent risk. **b.** Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs; rs9365492 and rs9355403 with the SNPs of gene providing Protection towards the Leprosy. Genotypes/Alleles in red color represent risk.

Table S1: Pathway analysis of PARK2 interacting protein with Cytokines studied by us in Indian population groups.

Table 1: Genotype interaction analysis of PARK2 SNPs with pro-/anti- inflammatory cytokines gene SNPs providing risk/protection towards Leprosy susceptibility

PARK2 (SNPs are within 63.8kb upstream gene region)		GENE	SNPs PROVIDING RISK			No. of Samples		Sig.	OR	95% C.I.for EXP(B)	
						Patients	Controls			Lower	Upper
rs9365492; Minor, Risk allele-C	rs9355403; Minor, Risk allele-A	IL-10	rs1800871 (-819); Minor, Risk allele-T	rs1554286 (intron 3 boundary); Minor, Risk allele-T	rs1800872 (-592); Minor, Risk allele-A						
TC+CC	GA+AA		CT+TT	TT	CA+AA	82	84	3.22E-05	1.997	1.441	2.767
		TGFBR2	rs2228048 (3' UTR Downstream); Minor, Risk allele-T	rs744751(3' UTR Downstream); Minor, Risk allele-G							
			CT+CC	GG		287	400	1.04E-02	1.293	1.062	1.575
		IL-6	rs1800797 (-718); Minor, Risk allele-G								
			GG			320	432	2.90E-03	1.333	1.103	1.611
		TNF	rs1800629 (-308); Minor, Risk allele-G	rs1800610 (Intron-1); Minor, Risk allele-G							
			GG	GG		311	420	2.06E-09	2.103	1.649	2.682
		BTNL2-DRA interval	rs3135365; Minor, Risk allele-C	rs7773756; Minor, Risk allele-T							
			CA+CC	CT+TT		272	269	1.22E-21	5.4	3.821	7.631
			SNPs PROVIDING PROTECTION								
TT	GG	LTA	rs13192469(13kb upstream); Minor, Risk allele-C	rs36221459 (-1409); Minor, Risk allele-DEL							
			TT	GTTT		240	571	3.56E-07	0.616	0.512	0.743
		IL-10RB2	rs3171425 (3' UTR); Major, Risk allele-G	rs7281762 (3' UTR Downstream); Minor, Risk allele-A							
			GA+AA	GA+GG		164	391	1.10E-05	0.61	0.489	0.76
		BAT1	rs2523504 (-603); Minor, Risk allele-T								
			CC			192	475	4.15E-05	0.645	0.523	0.795
		NFKBIL	rs2230365 (Exon-3); Minor, Risk allele-T								
			CC			195	486	1.01E-07	0.589	0.484	0.715
		TNF-LTB	rs769178 (Gene Downstream); Major, Risk allele-G								
			GT+TT			66	211	8.93E-05	0.546	0.404	0.739
		IL12B	rs2853694; Major, Risk allele-A								
			CA+CC			233	519	5.03E-04	0.705	0.579	0.858

Table 2: Combined interaction analysis of all the SNPs either providing protection or risk towards Leprosy susceptibility

a. Analysis of SNPs providing Protection

	No. of Samples		PARK2	PARK2	BAT1 Promoter	NFKBIL	LTA 13kb upstream	LTA Promoter	TNF-LTB	IL10RB	IL10RB	IL12B	Sig.	OR	95% C.I.for EXP(B)	
	Pat	Cont	rs9365492	rs9355403	rs2523504	rs2230365	rs13192469	rs36221459	rs769178	rs3171425	rs7281762	rs2853694			Lower	Upper
Alleles			T/C	G/A	C/T	C/T	T/C	GTTT/DEL	G/T	G/A	G/A	A/C				
Risk Allele			C	A	T	T	C	DEL	G	G	A	A				
TOTAL	10	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	1.15E-04	0.263	0.133	0.518
PB/HC	2	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	2.36E-03	0.111	0.027	0.458
MB/HC	NA															

b. Analysis of SNPs providing Risk

	No. of Samples		PARK2	PARK2	IL-10	IL-10	IL-10	IL-6	TGFBR2	TGFBR2	TNF (-308) Promoter	TNF intron1	BTNL2-DRA interval	BTNL2-DRA interval	Sig.	OR	95% C.I.for EXP(B)	
	Pat	Cont	rs9365492	rs9355403	rs1800871	rs1554286	rs1800872	rs1800797	rs2228048	rs744751	rs1800629	rs1800610	rs3135365	rs7773756			Lower	Upper
Alleles			T/C	G/A	C/T	C/T	C/A	G/A	C/T	G/A	G/A	G/A	A/C	C/T				
Risk Allele			C	A	T	T	A	G	T	G	G	G	C	T				
Total	57	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GA+AA	GG	GG	CA+CC	CT+TT	5.77E-06	2.543	1.699	3.806
PB/HC	32	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.73E-06	3.028	1.894	4.843
MB/HC	25	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.16E-03	2.133	1.29	3.528

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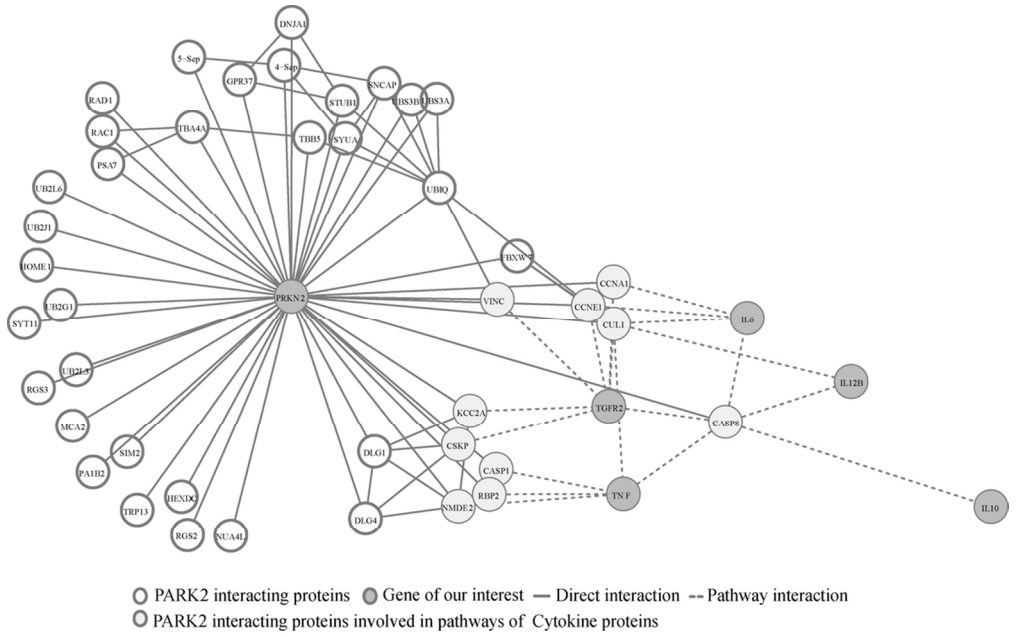
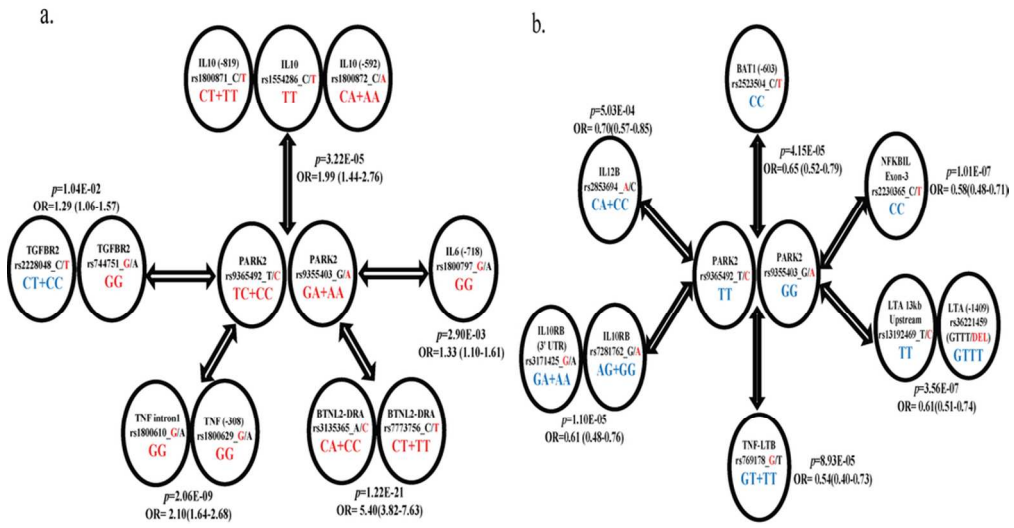


PARK2 Interaction Analysis: unfilled circles showing the PARK2 interacting proteins, Light grey circles showing protein links between the PARK2 interacting protein and 5 cytokines protein (dark grey circles) study by us in North India population
100x62mm (300 x 300 DPI)



a. Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs rs9365492 and rs9355403 with the SNPs of gens providing Risk toward the Leprosy. Genotypes/Alleles in red color represent risk. b. Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs; rs9365492 and rs9355403 with the SNPs of gene providing Protection towards the Leprosy. Genotypes/Alleles in red color represent risk.
89x46mm (300 x 300 DPI)

Table S1: Pathway analysis of PARK2 interacting protein with Cytokines studied by us in Indian population groups

PARK2-interacting proteins	Uniport_ID of PARK2 interacting proteins	PATHWAYS involving PARK2 interacting proteins and Cytokine genes	Cytokine genes	Databases used for Pathways retrieval
CUL1	Q13616	Adherens junction	TGFR2	KEGG
VINC	P18206	Adherens junction	TGFR2	KEGG
CUL1	Q13616	Amyotrophic lateral sclerosis (ALS)	TNF	KEGG
NMDE2	Q13224	Amyotrophic lateral sclerosis (ALS)	TNF	KEGG
CUL1	Q13616	Angiopoietin receptor Tie2-mediated signaling	TNF	Nci-Nature
CASP8	Q14790	Apoptosis	TNF	KEGG
CASP8	Q14790	caspase cascade in apoptosis	TNF	BioCarta
CASP8	Q14790	Caspase cascade in apoptosis	TNF	Nci-Nature
CASP1	P29466	caspase cascade in apoptosis	TNF	BioCarta
CASP1	P29466	Caspase cascade in apoptosis	TNF	Nci-Nature
CASP1	P29466	Cellular roles of Anthrax toxin	TNF	Nci-Nature
CASP8	Q14790	ceramide signaling pathway	TNF	BioCarta
CASP8	Q14790	Ceramide signaling pathway	TNF	Nci-Nature
CASP8	Q14790	Chagas disease	IL10, TGFR2, TNF	KEGG
CUL1	Q13616	Fc epsilon RI signaling pathway	TNF	KEGG
CASP8	Q14790	hiv-1 nef: negative effector of fas and tnf	TNF	BioCarta
CASP8	Q14790	HIV-1 Nef: Negative effector of Fas and TNF-alpha	TNF	Nci-Nature
CUL1	Q13616	IL6-mediated signaling events	IL6	Nci-Nature
CUL1	Q13616	Integrins in angiogenesis	TGFR2	Nci-Nature
CASP8	Q14790	Integrins in angiogenesis	TGFR2	Nci-Nature
VINC	P18206	Integrins in angiogenesis	TGFR2	Nci-Nature
CUL1	Q13616	LPA receptor mediated events	IL6	Nci-Nature

CUL1	Q13616	MAPK signaling pathway	TGFR2, TNF	KEGG
CUL1	Q13616	Natural killer cell mediated cytotoxicity	TNF	KEGG
RBP2	P49792	Signaling events mediated by HDAC Class I	TNF	Nci-Nature
CSKP	O14936	Syndecan-1-mediated signaling events	TGFR2	Nci-Nature
KCC2A	Q9UQM7	TGF-beta receptor signaling	TGFR2	Nci-Nature
CUL1	Q13616	TGF-beta signaling pathway	TGFR2, TNF	KEGG
CASP8	Q14790	TNF receptor signaling pathway	TNF	Nci-Nature
CASP8	Q14790	TNF signaling	TNF	Reactome
CASP8	Q14790	tnfr1 signaling pathway	TNF	BioCarta
CUL1	Q13616	Toll-like receptor signaling pathway	IL6, TNF, IL12B	KEGG
CASP8	Q14790	Toll-like receptor signaling pathway	IL6, TNF, IL12B	KEGG

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PARK2 and pro-/anti- inflammatory cytokine gene interactions contribute to the susceptibility to Leprosy – A Case/Control study of North Indian population

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Title: PARK2 and pro-/anti- inflammatory cytokine gene interactions contribute to the susceptibility to Leprosy – A Case/Control study of North Indian population

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Abstract

Objectives: Cytokines and related molecules in immune-response pathways seem important in deciding the outcome of the host-pathogen interactions towards different polar forms in Leprosy. Here we study the role of significant and functionally important SNPs in these genes, published independently from our research group, through combined interaction with an additional analysis of the *in-silico* network outcome, to understand how these impact the susceptibility towards the disease, leprosy.

Design: Study was designed to assess an overall combined contribution of significantly associated individual SNPs to reflect on epistatic interactions and their outcome in the form of the disease, leprosy. Further *in-silico* approach was adopted to carry out Protein-Protein interaction study between PARK2 and pro-/anti- inflammatory cytokines.

Setting: Population based case-control study involved the data of North India. Protein-Protein interaction networks were constructed using Cytoscape.

Participants: Study included the data available from 2305 Northern Indians samples (829 Leprosy Patients; 1476 Healthy controls), generated by our research group.

Primary and secondary outcome measures: For genotype interaction analysis, all possible genotype combinations between selected SNPs were used as an independent variable, using binary logistic regression with the forward likelihood ratio method, keeping the gender as a covariate.

Results: Interaction analysis between PARK2 and significant SNPs of anti-/pro- inflammatory cytokine genes, including (BAT1 to BTNL2-DR) spanning the HLA (6p21.3) region in a case-control comparison, showed that the combined analysis of: (i) PARK2, TNF, BTNL2-DR, IL10, IL-6 & TGFBR2 increased the risk towards leprosy (OR=2.54); (ii) PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B & IL10RB provided increased protection (OR=0.26) in comparison to their individual contribution.

Conclusions: Epistatic SNP-SNP interactions involving PARK2 and cytokine genes provide an additive risk towards leprosy susceptibility. Further, *in-silico* Protein-Protein Interaction of

PARK2 and important pro-/anti inflammatory molecules indicate that PARK2 is central to immune regulation, regulating the production of different cytokines upon infection.

Keywords: PARK2, pro-/anti- inflammatory cytokine genes, Leprosy, Gene interaction

Article Summary

Strengths and limitation of this study

- Many of the genetic studies lack replication in different population groups, explaining the heterogeneity in associated genes and genomic regions. This may be due to the complex nature of a disease. The complexity, however, could partly be delineated at genetic level by assessing the quantum of the contribution of different loci to the disease in a combined manner instead of their individual role.
- Our study highlights the importance of a combined effect of the important cytokine and other immune regulatory genes, whose combined effect in diverse genotype combinations, provide either increased risk or protection towards the complex genetic disease, Leprosy.
- Genetic interaction and an additional *in-silico* pathway analysis provided an overall perspective on how PARK2 gene product; parkin, acts as a centrally placed molecule, playing an important role in regulating different pathways of the immune response and susceptibility to Leprosy. This conclusion needs further support with future experiments *in vitro* or *in vivo* of T cell responses in different genetic backgrounds of the identified networks in this study.

Introduction

Leprosy caused by *Mycobacterium leprae* is a chronic infectious disease, characterized by clinically defined polar forms in which pathology and immunology are inextricably related, providing a critical model to explore the immuno-regulatory mechanisms in humans. At one pole, tuberculoid form is associated with a strong cell mediated immunity and T helper 1 (Th1) cytokine profile and at the other end of the spectrum, the lepromatous form is associated with a strong humoral response and T helper 2 (Th2) cytokine profile. Cytokines and other related molecules of the immunological pathways thus seem to be a part of significant group of candidates that are apparently critical for the host-pathogen interactions, where the outcome of the disease is majorly dependent on the host factors controlling the immune response, especially when *M. leprae* possesses lowest level of genetic diversity [1]. This is supported by various studies of familial clustering [2], twin studies [3], complex segregation analysis [4 5], test of analysis with the HLA genes [6] including recent genome-wide association studies, [7 8] and studies of several genes that modulate cell-mediated immunity, with a role in either susceptibility to leprosy per se or to leprosy types [9]. Various candidate gene studies and genome-wide approaches have implicated polymorphisms in cytokine genes, whose protein products are part of important immune modulatory molecules, playing a major role in influencing host-pathogen interactions and determine the outcome of many infectious and autoimmune diseases [10-16]. However, only a few observations have been replicated unequivocally in different population groups, suggesting the polygenic nature of the disease with a high degree of heterogeneity among different populations.

We, in the recent past, have studied various candidate genes of pro-/anti-inflammatory cytokines in two independent population groups, North and East India-Orissa; and found strong association with IL-10, IL-10RB, TGFBR2, IL-6 [14], IL-12B [17]. Fine-mapping of a specific 6p (HLA) chromosomal region revealed a significant association of important candidates, BAT1, LTA, TNF and BTNL2 [16]. In a subsequent study of the 6q chromosomal region, involving the overlapping regulatory domain of PARK2-PACRG genes, revealed an involvement of significant SNPs and presence of a differential LD structure in Indian populations as compared to Vietnamese [18]. The latter observation and the functional role of PARK2, as a ubiquitin ligase,

has recently been shown in providing resistance to intracellular pathogens [19] through ubiquitin mediated autophagy. Further the involvement of parkin in regulating production of cytokines upon infection [20], indeed provides strong hint for any functional variation in the gene having profound effect in modulating the expression of the immune-regulatory genes. The importance of all the studied genes [14-18] in the network of immune-response necessitated the analysis of an interaction between these genes as a whole to understand their contribution together towards the susceptibility of the complex disease, Leprosy; where the outcome of the infection in all probabilities depends on the nature of gene interactions between the genes with the potential of contributing to the immune pathology.

The aim, therefore, of this study was to assess an overall interaction between the significant and functionally important SNPs studied in a case-control comparison of the samples from New Delhi, in Northern India; where most of these SNPs were replicated in an unrelated East Indian-Orissa population. These included for an overall interaction the PARK2 gene significant SNPs [18] with the significant SNPs of anti-inflammatory cytokine genes (IL-10, IL-10RB, TGFBR2, IL-6) [14], pro-inflammatory cytokine genes (TNF-alpha, LT-alpha, IL-12B) and the genes spanning the HLA region of the chromosome 6p21.3 i.e., BAT1 to BTNL2-DR [16 17] to evaluate their combined contribution towards the outcome of the complex infectious disease; Leprosy.

Methods

The study involved the revisit of our published work on individual candidate genes and regions, studied in North Indian population groups in case-control comparison, for a combined genotype interaction and for *in-silico* protein-protein interaction and network analysis. The data compiled was of 2305 samples from Northern India (including 829 Leprosy patients and 1476 unrelated healthy control subjects from North India) [14 16-18] with a complete coverage of genes belonging to pro-, anti-inflammatory cytokines, selected HLA regions in 6p21.3 and common regulatory region of PARK2/PACRG genes located at 6q26 region.

The patients' group was classified according to the WHO guidelines. Individual was regarded as having leprosy if he or she showed skin lesion consistent with leprosy and with definite sensory

loss, with or without thickened nerves and positive skin smears test. Further patients were classified as pauci-bacillary (PB) or multi-bacillary (MB) according to the Ridley and Jopling criteria [21], including 421 Pauci-bacillary patients and 408 Multi-bacillary patients, with a mean age of 32.30±3.2 years (range 6-80 years). All these patients were under treatment with multidrug therapy (MDT) specific for multibacillary (MB) and paucibacillary (PB) leprosy, as recommended by the World Health Organization.

For genotype interaction analysis, all possible genotype combinations between selected SNPs (pairwise or multiple genes) were ascertained from a MassArray platform for the given genotypes of SNPs. However, only the combinations of significantly associated SNP genotypes were presented in the Ms for convenience. These interactions were tested using binary logistic regression with the forward likelihood ratio based selection method considering all variables independently and keeping gender as a covariate. In this selection method, entry testing based on the significance of the score statistics and removal testing based on the probability of a likelihood ratio statistics were applied. Furthermore, in multiple gene interaction analysis, all interactions with either risk or protection were combined against other interactions to observe the overall effect of all risk versus protective interactions. These analyses were performed using statistical software package SPSS V.17.0 (SPSS, Chicago III, Illinois, USA) for Windows. *P* Value was considered significant at and below 0.05.

In-silico approach to assess the network of the genes in a Protein-Protein Interaction (PPI) of PARK2, using Agile Protein Interaction Database (APID), a comprehensive resource for protein interaction data, automatically accessed by Cytoscape [22] through the dedicated plugin APID2NET [23], was carried out to understand the involvement of the studied interactome. APID integrates in a single web-based tool all known experimentally validated PPI from BIND [24], BioGRID [25], DIP [26], HPRD [27], IntAct [28] and MINT [29] databases.

Results

The interaction analysis carried out between PARK2 gene regulatory region SNPs (rs9365492 & rs9355403) [18] and SNPs of the anti-inflammatory cytokines [14] provided a significant risk towards the leprosy susceptibility; combining individually with SNPs of IL-10 (OR=1.99), IL-6 (OR=1.33) and TGFBR2 (OR=1.29) cytokine genes. However, with IL10RB (receptor beta), the

result showed a significant protection towards the disease (OR=0.61). Similar analysis between PARK2 SNPs with pro-inflammatory cytokine genes TNF-alpha and BTNL2-DRA interval (showing strong LD with the BTNL2 promoter SNPs) [16] provided a significant risk towards leprosy susceptibility with OR=2.10 and OR=5.40, respectively. However, the SNPs of BAT-1, NFKABIL1, LTA, TNF-LTB, IL12B [16 17] provided a significant protection towards leprosy with OR=0.65, OR=0.58, OR=0.61, OR=0.54 and OR=0.71, respectively (Table 1, Figure S1).

In the second step of combined interaction analysis with all the genes, either providing protection or risk towards leprosy, showed that the combined genotypic interaction analysis of the SNP loci PARK2, TNF, BTNL2-DR, IL10, IL-6 and TGFBR2, further increased the risk of leprosy (OR=2.54); and a similar combined analysis for loci PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB, increased protection towards leprosy (OR=0.26), in comparison to their individual contribution (Table 2 (a, b)). Dividing the patients in pauci-bacillary (PB) and multi-bacillary (MB) subtypes of leprosy revealed PB subtype to carry a higher Risk (OR= 3.02) and Protection (OR= 0.11) towards Leprosy in comparison to MB subtype, for respective combinations.

We further did *in-silico* analysis to identify the Protein-Protein Interaction (PPI) of PARK2. We used APID2NET and Cytoscape tools for PARK2 interaction Data retrieval, providing a total of 43 PARK2 interacting proteins. However, the result did not provide any direct interaction of the PARK2 with the cytokines studied by us in North Indian population [14 16-18]. Further, we considered 43 PARK2 interacting proteins for pathways analysis by using KEGG , BioCarta, Nci-Nature and Reactome tools, resulting these 43 proteins to be involved in 253 different pathways (without removing overlapping pathways). Similarly, in the second step of pathway analysis we considered 11 cytokine proteins studied by us in North Indian population [14 16-18], the results revealed the involvement of 5 cytokine proteins; IL12B, IL6, TNF, TGFR2 & IL10 in 94 pathways, not involving BTNL2, BAT1, NFKBIL, LTA, IL10RB2 & BTNL2-DR in any pathways. Comparing both pathways; 253, PARK2 interacting proteins pathways and 94, cytokine proteins pathways; reveals 27 commonly involved pathways, via CASP8, CUL1, CCNE1 & CCNA proteins, involving only 5 (IL12B, IL6, TNF, TGFR2 & IL10) out of 11 cytokine proteins studied in North Indian population (Figure-1), connecting majorly through Toll-like receptor signaling pathways (Figure 1, Table S1).

Discussion

Leprosy, an ideal model of a chronic human complex infectious disease, provides an opportunity to dissect the components of the host dependent polygenic susceptibility to this disease. Many loci have been shown to be individually associated and providing the risk towards the disease; justifying to find out interesting gene–gene interactions at different risk loci which may prove to provide a strong association towards the disease susceptibility. In order to understand the role of multiple genes together, an interaction analysis was carried out between the genotype status of functionally different variants of different genetic loci involved in immune response, with an expected combined effect on the outcome of the disease in different polar forms of the disease.

Considering the above facts we first carried out pair-wise interaction analysis of PARK2 gene with pro-/anti- inflammatory cytokine genes (Table 1) followed by multiple gene interaction analysis (Table 2). Analysis of PARK2 with TNF, BTNL2-DR, IL10, IL-6 and TGFBR2, showed an increased risk towards leprosy (OR=2.54 (1.69-3.80), p=5.77e-06); while as the combined analysis of PARK2 with BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB, showed protection towards the disease (OR=0.26 (0.13-0.51), p=1.15e-04). PARK2, encoding E3 ubiquitin ligase protein-parkin, has been shown to be involved in the cellular ubiquitination metabolism [30], providing resistance to intracellular pathogen via ubiquitin mediated autophagy [19], essentially shown to be involved in the host responses to *M. leprae* [31] and for pathogenesis of the disease [8 32]. Recently, parkin protein has shown to be involved to respond to infection in a regulated way by producing important cytokines [20], suppressing molecules that limit pro-inflammatory- IL-2 [33], TNF- alpha cytokine production and enhancing the production of anti-inflammatory cytokines, IL-4, IL-10, and IL-13 [34-39]. All these observations indicate the *in-vivo* importance of PARK2 gene product-parkin to be centrally involved in regulating the production of critical cytokines during immune response against the invading mycobacterium and justifying our study, where combination of risk genotype at different loci of important immune response gene with PARK2 provide increased and significant risk towards this complex disease. These interesting results of gene-gene interaction analysis, suggest seeing the *in-vivo* effect of the invading mycobacterium in future, where immune

response to specific antigens is assessed in cells with different background of important variations in the PARK2 promoter region followed by the effect on the expression levels of pro-/anti- inflammatory cytokines.

An *in-silico* approach to understand the role of immune-regulatory protein-protein interaction (PPI) between PARK2 and other cytokine genes, an indirect interaction was observed between PARK2 and IL12B, IL6, TNF, TGFR2 & IL10 genes. All these interactions were found to be connected with Toll-like receptor signaling pathway (Table S1). As already known that the polymorphisms in different TLRs, an important molecules of innate immune response are associated with Leprosy and its subtypes [7 40-44], influencing recognition of *M. Leprae.* A simultaneous involvement of PARK2, a ubiqutin ligase protein involved in innate immunity by modulating the production of important cytokines, including IL6 [20]; hints at the involvement of all these important molecules to be inter-connected through a TLR receptor signaling pathway to fight against the invading.

The above interaction and pathway analysis allows us to propose that the complex genetic background is the predominant factor for the outcome of the disease, where the combined effect of the variant risk alleles of the PARK2 gene, responsible for affecting transcription binding site and lowering the expression of the reporter gene by *in-vitro* experiment [18], along with the risk alleles of the anti-inflammatory cytokines genes - IL-10, IL-6, TGFBR2, responsible for lowering the CMI response towards the invading bacteria and pro-inflammatory cytokines - TNF-alpha; is responsible in providing highly significant risk towards leprosy. The study opens a way for future *in-vivo* work of immune-response read outs in complex variant genomic backgrounds to understand the wide gap in understanding the balance in the network of all the immune regulatory molecules operational in providing either susceptibility or resistance towards disease.

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Data sharing statement: Extra data is made available by contacting corresponding author at Email ID: bamezai@hotmail.com.

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Patient consent: Obtained.

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Figure 1: PARK2 Interaction Analysis: unfilled circles showing the PARK2 interacting proteins, Light grey circles showing protein links between the PARK2 interacting protein and 5 cytokines protein (dark grey circles) study by us in North India population

Figure S1: a. Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs rs9365492 and rs9355403 with the SNPs of gens providing Risk toward the Leprosy. Genotypes/Alleles in red color represent risk. **b.** Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs; rs9365492 and rs9355403 with the SNPs of gene providing Protection towards the Leprosy. Genotypes/Alleles in red color represent risk.

Table S1: Pathway analysis of PARK2 interacting protein with Cytokines studied by us in Indian population groups.

Table 1: Genotype interaction analysis of PARK2 SNPs with pro-/anti- inflammatory cytokines gene SNPs providing risk/protection towards Leprosy susceptibility

rs9365492; Minor, Risk allele-C	rs9355403; Minor, Risk allele-A	IL-10	rs1800871 (-819); Minor, Risk allele-T	rs1554286 (intron 3 boundary); Minor, Risk allele-T	rs1800872 (-592); Minor, Risk allele-A						
TC+CC	GA+AA		CT+TT	TT	CA+AA	82	84	3.22E-05	1.997	1.441	2.767
		TGFBR2	rs2228048 (3' UTR Downstream); Minor, Risk allele-T	rs744751(3' UTR Downstream); Minor, Risk allele-G							
			CT+CC	GG		287	400	1.04E-02	1.293	1.062	1.575
		IL-6	rs1800797 (-718); Minor, Risk allele-G								
			GG			320	432	2.90E-03	1.333	1.103	1.611
		TNF	rs1800629 (-308); Minor, Risk allele-G	rs1800610 (Intron-1); Minor, Risk allele-G							
			GG	GG		311	420	2.06E-09	2.103	1.649	2.682
		BTNL2-DRA interval	rs3135365; Minor, Risk allele-C	rs7773756; Minor, Risk allele-T							
			CA+CC	CT+TT		272	269	1.22E-21	5.4	3.821	7.631
TT	GG	LTA	rs13192469(13kb upstream); Minor, Risk allele-C	rs36221459 (-1409); Minor, Risk allele-DEL							
			TT	GTTT		240	571	3.56E-07	0.616	0.512	0.743
		IL-10RB2	rs3171425 (3' UTR); Major, Risk allele-G	rs7281762 (3' UTR Downstream); Minor, Risk allele-A							
			GA+AA	GA+GG		164	391	1.10E-05	0.61	0.489	0.76
		BAT1	rs2523504 (-603); Minor, Risk allele-T								
			CC			192	475	4.15E-05	0.645	0.523	0.795
		NFKBIL	rs2230365 (Exon-3); Minor, Risk allele-T								
			CC			195	486	1.01E-07	0.589	0.484	0.715
		TNF-LTB	rs769178 (Gene Downstream); Major, Risk allele-G								
			GT+TT			66	211	8.93E-05	0.546	0.404	0.739
		IL12B	rs2853694; Major, Risk allele-A								
			CA+CC			233	519	5.03E-04	0.705	0.579	0.858

Table 2: Combined interaction analysis of all the SNPs either providing protection or risk towards Leprosy susceptibility

a. Analysis of SNPs providing Protection

	Pat	Cont	rs9365492	rs9355403	rs2523504	rs2230365	rs13192469	rs36221459	rs769178	rs3171425	rs7281762	rs2853694			Lower	Upper
			T/C	G/A	C/T	C/T	T/C	GTTT/DEL	G/T	G/A	G/A	A/C				
			C	A	T	T	C	DEL	G	G	A	A				
	10	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	1.15E-04	0.263	0.133	0.518
	2	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	2.36E-03	0.111	0.027	0.458
	NA															

b. Analysis of SNPs providing Risk

	Pat	Cont	rs9365492	rs9355403	rs1800871	rs1554286	rs1800872	rs1800797	rs2228048	rs744751	rs1800629	rs1800610	rs3135365	rs7773756			Lower	Upper
			T/C	G/A	C/T	C/T	C/A	G/A	C/T	G/A	G/A	G/A	A/C	C/T				
			C	A	T	T	A	G	T	G	G	G	C	T				
	57	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GA+AA	GG	GG	CA+CC	CT+TT	5.77E-06	2.543	1.699	3.806
	32	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.73E-06	3.028	1.894	4.843
	25	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.16E-03	2.133	1.29	3.528

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Title: PARK2 and pro-/anti- inflammatory cytokine gene interactions contribute to the susceptibility to Leprosy – A Case/Control study of North Indian population

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Abstract

Objectives: Cytokines and related molecules in immune-response pathways seem important in deciding the outcome of the host-pathogen interactions towards different polar forms in Leprosy. Here we study the role of significant and functionally important SNPs in these genes, published independently from our research group, through combined interaction with an additional analysis of the *in-silico* network outcome, to understand how these impact the susceptibility towards the disease, leprosy.

Design: Study was designed to assess an overall combined contribution of significantly associated individual SNPs to reflect on epistatic interactions and their outcome in the form of the disease, leprosy. Further *in-silico* approach was adopted to carry out Protein-Protein interaction study between PARK2 and pro-/anti- inflammatory cytokines.

Setting: Population based case-control study involved the data of North India. Protein-Protein interaction networks were constructed using Cytoscape.

Participants: Study included the data available from 2305 Northern Indians samples (829 Leprosy Patients; 1476 Healthy controls), generated by our research group.

Primary and secondary outcome measures: For genotype interaction analysis, all possible genotype combinations between selected SNPs were used as an independent variable, using binary logistic regression with the forward likelihood ratio method, keeping the gender as a covariate.

Results: Interaction analysis between PARK2 and significant SNPs of anti-/pro- inflammatory cytokine genes, including (BAT1 to BTNL2-DR) spanning the HLA (6p21.3) region in a case-control comparison, showed that the combined analysis of: (i) PARK2, TNF, BTNL2-DR, IL10, IL-6 & TGFBR2 increased the risk towards leprosy (OR=2.54); (ii) PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B & IL10RB provided increased protection (OR=0.26) in comparison to their individual contribution.

Conclusions: Epistatic SNP-SNP interactions involving PARK2 and cytokine genes provide an additive risk towards leprosy susceptibility. Further, *in-silico* Protein-Protein Interaction of PARK2 and important pro-/anti inflammatory molecules indicate that PARK2 is central to immune regulation, regulating the production of different cytokines upon infection.

Keywords: PARK2, pro-/anti- inflammatory cytokine genes, Leprosy, Gene interaction

Article Summary

Strengths and limitation of this study

- Many of the genetic studies lack replication in different population groups, explaining the heterogeneity in associated genes and genomic regions. This may be due to the complex nature of a disease. The complexity, however, could partly be delineated at genetic level by assessing the quantum of the contribution of different loci to the disease in a combined manner instead of their individual role.

- Our study highlights the importance of a combined effect of the important cytokine and other immune regulatory genes, whose combined effect in diverse genotype combinations, provide either increased risk or protection towards the complex genetic disease, Leprosy.
- Genetic interaction and an additional *in-silico* pathway analysis provided an overall perspective on how PARK2 gene product; parkin, acts as a centrally placed molecule, playing an important role in regulating different pathways of the immune response and susceptibility to Leprosy. This conclusion needs further support with future experiments *in vitro* or *in vivo* of T cell responses in different genetic backgrounds of the identified networks in this study.

Introduction

Leprosy caused by *Mycobacterium leprae* is a chronic infectious disease, characterized by clinically defined polar forms in which pathology and immunology are inextricably related, providing a critical model to explore the immuno-regulatory mechanisms in humans. At one pole, tuberculoid form is associated with a strong cell mediated immunity and T helper 1 (Th1) cytokine profile and at the other end of the spectrum, the lepromatous form is associated with a strong humoral response and T helper 2 (Th2) cytokine profile. Cytokines and other related molecules of the immunological pathways thus seem to be a part of significant group of candidates that are apparently critical for the host-pathogen interactions, where the outcome of the disease is majorly dependent on the host factors controlling the immune response, especially when *M. leprae* possesses lowest level of genetic diversity [1]. This is supported by various studies of familial clustering [2], twin studies [3], complex segregation analysis [4 5], test of analysis with the HLA genes [6] including recent genome-wide association studies, [7 8] and studies of several genes that modulate cell-mediated immunity, with a role in either susceptibility to leprosy per se or to leprosy types [9]. Various candidate gene studies and genome-wide approaches have implicated polymorphisms in cytokine genes, whose protein products are part of important immune modulatory molecules, playing a major role in influencing host-pathogen interactions and determine the outcome of many infectious and autoimmune diseases [10-16]. However, only a few observations have been replicated unequivocally in different population

groups, suggesting the polygenic nature of the disease with a high degree of heterogeneity among different populations.

We, in the recent past, have studied various candidate genes of pro-/anti-inflammatory cytokines in two independent population groups, North and East India-Orissa; and found strong association with IL-10, IL-10RB, TGFBR2, IL-6 [14], IL-12B [17]. Fine-mapping of a specific 6p (HLA) chromosomal region revealed a significant association of important candidates, BAT1, LTA, TNF and BTNL2 [16]. In a subsequent study of the 6q chromosomal region, involving the overlapping regulatory domain of PARK2-PACRG genes, revealed an involvement of significant SNPs and presence of a differential LD structure in Indian populations as compared to Vietnamese [18]. The latter observation and the functional role of PARK2, as a ubiquitin ligase, has recently been shown in providing resistance to intracellular pathogens [19] through ubiquitin mediated autophagy. Further the involvement of parkin in regulating production of cytokines upon infection [20], indeed provides strong hint for any functional variation in the gene having profound effect in modulating the expression of the immune-regulatory genes. The importance of all the studied genes [14-18] in the network of immune-response necessitated the analysis of an interaction between these genes as a whole to understand their contribution together towards the susceptibility of the complex disease, Leprosy; where the outcome of the infection in all probabilities depends on the nature of gene interactions between the genes with the potential of contributing to the immune pathology.

The aim, therefore, of this study was to assess an overall interaction between the significant and functionally important SNPs studied in a case-control comparison of the samples from New Delhi, in Northern India; where most of these SNPs were replicated in an unrelated East Indian-Orissa population. These included for an overall interaction the PARK2 gene significant SNPs [18] with the significant SNPs of anti-inflammatory cytokine genes (IL-10, IL-10RB, TGFBR2, IL-6) [14], pro-inflammatory cytokine genes (TNF-alpha, LT-alpha, IL-12B) and the genes spanning the HLA region of the chromosome 6p21.3 i.e., BAT1 to BTNL2-DR [16 17] to evaluate their combined contribution towards the outcome of the complex infectious disease; Leprosy.

Methods

The study involved the revisit of our published work on individual candidate genes and regions, studied in North Indian population groups in case-control comparison, for a combined genotype interaction and for *in-silico* protein-protein interaction and network analysis. The data compiled was of 2305 samples from Northern India (including 829 Leprosy patients and 1476 unrelated healthy control subjects from North India) [14 16-18] with a complete coverage of genes belonging to pro-, anti-inflammatory cytokines, selected HLA regions in 6p21.3 and common regulatory region of PARK2/PACRG genes located at 6q26 region.

The patients' group was classified according to the WHO guidelines. Individual was regarded as having leprosy if he or she showed skin lesion consistent with leprosy and with definite sensory loss, with or without thickened nerves and positive skin smears test. Further patients were classified as pauci-bacillary (PB) or multi-bacillary (MB) according to the Ridley and Jopling criteria [21], including 421 Pauci-bacillary patients and 408 Multi-bacillary patients, with a mean age of 32.30±3.2 years (range 6-80 years). All these patients were under treatment with multidrug therapy (MDT) specific for multibacillary (MB) and paucibacillary (PB) leprosy, as recommended by the World Health Organization.

For genotype interaction analysis, all possible genotype combinations between selected SNPs (pairwise or multiple genes) were ascertained from a MassArray platform for the given genotypes of SNPs. However, only the combinations of significantly associated SNP genotypes were presented in the Ms for convenience. These interactions were tested using binary logistic regression with the forward likelihood ratio based selection method considering all variables independently and keeping gender as a covariate. In this selection method, entry testing based on the significance of the score statistics and removal testing based on the probability of a likelihood ratio statistics were applied. Furthermore, in multiple gene interaction analysis, all interactions with either risk or protection were combined against other interactions to observe the overall effect of all risk versus protective interactions. These analyses were performed using statistical software package SPSS V.17.0 (SPSS, Chicago III, Illinois, USA) for Windows. *P* Value was considered significant at and below 0.05.

In-silico approach to assess the network of the genes in a Protein-Protein Interaction (PPI) of PARK2, using Agile Protein Interaction Database (APID), a comprehensive resource for protein interaction data, automatically accessed by Cytoscape [22] through the dedicated plugin APID2NET [23], was carried out to understand the involvement of the studied interactome. APID integrates in a single web-based tool all known experimentally validated PPI from BIND [24], BioGRID [25], DIP [26], HPRD [27], IntAct [28] and MINT [29] databases.

Results

The interaction analysis carried out between PARK2 gene regulatory region SNPs (rs9365492 & rs9355403) [18] and SNPs of the anti-inflammatory cytokines [14] provided a significant risk towards the leprosy susceptibility; combining individually with SNPs of IL-10 (OR=1.99), IL-6 (OR=1.33) and TGFBR2 (OR=1.29) cytokine genes. However, with IL10RB (receptor beta), the result showed a significant protection towards the disease (OR=0.61). Similar analysis between PARK2 SNPs with pro-inflammatory cytokine genes TNF-alpha and BTNL2-DRA interval (showing strong LD with the BTNL2 promoter SNPs) [16] provided a significant risk towards leprosy susceptibility with OR=2.10 and OR=5.40, respectively. However, the SNPs of BAT-1, NFKABIL1, LTA, TNF-LTB, IL12B [16 17] provided a significant protection towards leprosy with OR=0.65, OR=0.58, OR=0.61, OR=0.54 and OR=0.71, respectively (Table 1, Figure S1).

In the second step of combined interaction analysis with all the genes, either providing protection or risk towards leprosy, showed that the combined genotypic interaction analysis of the SNP loci PARK2, TNF, BTNL2-DR, IL10, IL-6 and TGFBR2, further increased the risk of leprosy (OR=2.54); and a similar combined analysis for loci PARK2, BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB, increased protection towards leprosy (OR=0.26), in comparison to their individual contribution (Table 2 (a, b)). Dividing the patients in pauci-bacillary (PB) and multi-bacillary (MB) subtypes of leprosy revealed PB subtype to carry a higher Risk (OR= 3.02) and Protection (OR= 0.11) towards Leprosy in comparison to MB subtype, for respective combinations.

We further did *in-silico* analysis to identify the Protein-Protein Interaction (PPI) of PARK2. We used APID2NET and Cytoscape tools for PARK2 interaction Data retrieval, providing a total of 43 PARK2 interacting proteins. However, the result did not provide any direct interaction of the

PARK2 with the cytokines studied by us in North Indian population [14 16-18]. Further, we considered 43 PARK2 interacting proteins for pathways analysis by using KEGG , BioCarta, Nci-Nature and Reactome tools, resulting these 43 proteins to be involved in 253 different pathways (without removing overlapping pathways). Similarly, in the second step of pathway analysis we considered 11 cytokine proteins studied by us in North Indian population [14 16-18], the results revealed the involvement of 5 cytokine proteins; IL12B, IL6, TNF, TGFR2 & IL10 in 94 pathways, not involving BTNL2, BAT1, NFKBIL, LTA, IL10RB2 & BTNL2-DR in any pathways. Comparing both pathways; 253, PARK2 interacting proteins pathways and 94, cytokine proteins pathways; reveals 27 commonly involved pathways, via CASP8, CUL1, CCNE1 & CCNA proteins, involving only 5 (IL12B, IL6, TNF, TGFR2 & IL10) out of 11 cytokine proteins studied in North Indian population (Figure-1), connecting majorly through Toll-like receptor signaling pathways (Figure 1, Table S1).

Discussion

Leprosy, an ideal model of a chronic human complex infectious disease, provides an opportunity to dissect the components of the host dependent polygenic susceptibility to this disease. Many loci have been shown to be individually associated and providing the risk towards the disease; justifying to find out interesting gene–gene interactions at different risk loci which may prove to provide a strong association towards the disease susceptibility. In order to understand the role of multiple genes together, an interaction analysis was carried out between the genotype status of functionally different variants of different genetic loci involved in immune response, with an expected combined effect on the outcome of the disease in different polar forms of the disease.

Considering the above facts we first carried out pair-wise interaction analysis of PARK2 gene with pro-/anti- inflammatory cytokine genes (Table 1) followed by multiple gene interaction analysis (Table 2). Analysis of PARK2 with TNF, BTNL2-DR, IL10, IL-6 and TGFBR2, showed an increased risk towards leprosy (OR=2.54 (1.69-3.80), p=5.77e-06); while as the combined analysis of PARK2 with BAT1, NFKBIL1, LTA, TNF-LTB, IL12B and IL10RB, showed protection towards the disease (OR=0.26 (0.13-0.51), p=1.15e-04). PARK2, encoding E3 ubiquitin ligase protein-parkin, has been shown to be involved in the cellular ubiquitination

metabolism [30], providing resistance to intracellular pathogen via ubiquitin mediated autophagy [19], essentially shown to be involved in the host responses to *M. leprae* [31] and for pathogenesis of the disease [8 32]. Recently, parkin protein has shown to be involved to respond to infection in a regulated way by producing important cytokines [20], suppressing molecules that limit pro-inflammatory- IL-2 [33], TNF- alpha cytokine production and enhancing the production of anti-inflammatory cytokines, IL-4, IL-10, and IL-13 [34-39]. All these observations indicate the *in-vivo* importance of PARK2 gene product-parkin to be centrally involved in regulating the production of critical cytokines during immune response against the invading mycobacterium and justifying our study, where combination of risk genotype at different loci of important immune response gene with PARK2 provide increased and significant risk towards this complex disease. These interesting results of gene-gene interaction analysis, suggest seeing the *in-vivo* effect of the invading mycobacterium in future, where immune response to specific antigens is assessed in cells with different background of important variations in the PARK2 promoter region followed by the effect on the expression levels of pro-/anti- inflammatory cytokines.

An *in-silico* approach to understand the role of immune-regulatory protein-protein interaction (PPI) between PARK2 and other cytokine genes, an indirect interaction was observed between PARK2 and IL12B, IL6, TNF, TGFR2 & IL10 genes. All these interactions were found to be connected with Toll-like receptor signaling pathway (Table S1). As already known that the polymorphisms in different TLRs, an important molecules of innate immune response are associated with Leprosy and its subtypes [7 40-44], influencing recognition of *M. Leprae.* A simultaneous involvement of PARK2, a ubiqutin ligase protein involved in innate immunity by modulating the production of important cytokines, including IL6 [20]; hints at the involvement of all these important molecules to be inter-connected through a TLR receptor signaling pathway to fight against the invading.

The above interaction and pathway analysis allows us to propose that the complex genetic background is the predominant factor for the outcome of the disease, where the combined effect of the variant risk alleles of the PARK2 gene, responsible for affecting transcription binding site and lowering the expression of the reporter gene by *in-vitro* experiment [18], along with the risk

alleles of the anti-inflammatory cytokines genes - IL-10, IL-6, TGFBR2, responsible for lowering the CMI response towards the invading bacteria and pro-inflammatory cytokines - TNF-alpha; is responsible in providing highly significant risk towards leprosy. The study opens a way for future *in-vivo* work of immune-response read outs in complex variant genomic backgrounds to understand the wide gap in understanding the balance in the network of all the immune regulatory molecules operational in providing either susceptibility or resistance towards disease.

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Figure 1: PARK2 Interaction Analysis: unfilled circles showing the PARK2 interacting proteins, Light grey circles showing protein links between the PARK2 interacting protein and 5 cytokines protein (dark grey circles) study by us in North India population

Figure S1: a. Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs rs9365492 and rs9355403 with the SNPs of gens providing Risk toward the Leprosy. Genotypes/Alleles in red color represent risk. **b.** Schematic representation of the genotypic interaction analysis of PARK2 gene regulatory region SNPs; rs9365492 and rs9355403 with the SNPs of gene providing Protection towards the Leprosy. Genotypes/Alleles in red color represent risk.

Table S1: Pathway analysis of PARK2 interacting protein with Cytokines studied by us in Indian population groups.

Table 1: Genotype interaction analysis of PARK2 SNPs with pro-/anti- inflammatory cytokines gene SNPs providing risk/protection towards Leprosy susceptibility

PARK2 (SNPs are within 63.8kb upstream gene region)		GENE	SNPs PROVIDING RISK			No. of Samples		Sig.	OR	95% C.I.for EXP(B)	
						Patients	Controls			Lower	Upper
rs9365492; Minor, Risk allele-C	rs9355403; Minor, Risk allele-A	IL-10	rs1800871 (-819); Minor, Risk allele-T	rs1554286 (intron 3 boundary); Minor, Risk allele-T	rs1800872 (-592); Minor, Risk allele-A						
TC+CC	GA+AA		CT+TT	TT	CA+AA	82	84	3.22E-05	1.997	1.441	2.767
		TGFBR2	rs2228048 (3' UTR Downstream); Minor, Risk allele-T	rs744751(3' UTR Downstream); Minor, Risk allele-G							
			CT+CC	GG		287	400	1.04E-02	1.293	1.062	1.575
		IL-6	rs1800797 (-718); Minor, Risk allele-G								
			GG			320	432	2.90E-03	1.333	1.103	1.611
		TNF	rs1800629 (-308); Minor, Risk allele-G	rs1800610 (Intron-1); Minor, Risk allele-G							
			GG	GG		311	420	2.06E-09	2.103	1.649	2.682
		BTNL2-DRA interval	rs3135365; Minor, Risk allele-C	rs7773756; Minor, Risk allele-T							
			CA+CC	CT+TT		272	269	1.22E-21	5.4	3.821	7.631
			SNPs PROVIDING PROTECTION								
TT	GG	LTA	rs13192469(13kb upstream); Minor, Risk allele-C	rs36221459 (-1409); Minor, Risk allele-DEL							
			TT	GTTT		240	571	3.56E-07	0.616	0.512	0.743
		IL-10RB2	rs3171425 (3' UTR); Major, Risk allele-G	rs7281762 (3' UTR Downstream); Minor, Risk allele-A							
			GA+AA	GA+GG		164	391	1.10E-05	0.61	0.489	0.76
		BAT1	rs2523504 (-603); Minor, Risk allele-T								
			CC			192	475	4.15E-05	0.645	0.523	0.795
		NFKBIL	rs2230365 (Exon-3); Minor, Risk allele-T								
			CC			195	486	1.01E-07	0.589	0.484	0.715
		TNF-LTB	rs769178 (Gene Downstream); Major, Risk allele-G								
			GT+TT			66	211	8.93E-05	0.546	0.404	0.739
		IL12B	rs2853694; Major, Risk allele-A								
			CA+CC			233	519	5.03E-04	0.705	0.579	0.858

Table 2: Combined interaction analysis of all the SNPs either providing protection or risk towards Leprosy susceptibility

a. Analysis of SNPs providing Protection

	No. of Samples		PARK2	PARK2	BAT1 Promoter	NFKBIL	LTA 13kb upstream	LTA Promoter	TNF-LTB	IL10RB	IL10RB	IL12B	Sig.	OR	95% C.I.for EXP(B)	
	Pat	Cont	rs9365492	rs9355403	rs2523504	rs2230365	rs13192469	rs36221459	rs769178	rs3171425	rs7281762	rs2853694			Lower	Upper
Alleles			T/C	G/A	C/T	C/T	T/C	GTTT/DEL	G/T	G/A	G/A	A/C				
Risk Allele			C	A	T	T	C	DEL	G	G	A	A				
TOTAL	10	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	1.15E-04	0.263	0.133	0.518
PB/HC	2	59	TT	GG	CC	CC	TT	GTTT	GT+TT	GA+AA	GA+GG	CA+CC	2.36E-03	0.111	0.027	0.458
MB/HC	NA															

b. Analysis of SNPs providing Risk

	No. of Samples		PARK2	PARK2	IL-10	IL-10	IL-10	IL-6	TGFBR2	TGFBR2	TNF (-308) Promoter	TNF intron1	BTNL2-DRA interval	BTNL2-DRA interval	Sig.	OR	95% C.I.for EXP(B)	
	Pat	Cont	rs9365492	rs9355403	rs1800871	rs1554286	rs1800872	rs1800797	rs2228048	rs744751	rs1800629	rs1800610	rs3135365	rs7773756			Lower	Upper
Alleles			T/C	G/A	C/T	C/T	C/A	G/A	C/T	G/A	G/A	G/A	A/C	C/T				
Risk Allele			C	A	T	T	A	G	T	G	G	G	C	T				
Total	57	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GA+AA	GG	GG	CA+CC	CT+TT	5.77E-06	2.543	1.699	3.806
PB/HC	32	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.73E-06	3.028	1.894	4.843
MB/HC	25	44	TC+CC	GA+AA	CT+TT	CT+CC	CA+AA	GG	CT+CC	GG	GG	GG	CA+CC	CT+TT	3.16E-03	2.133	1.29	3.528

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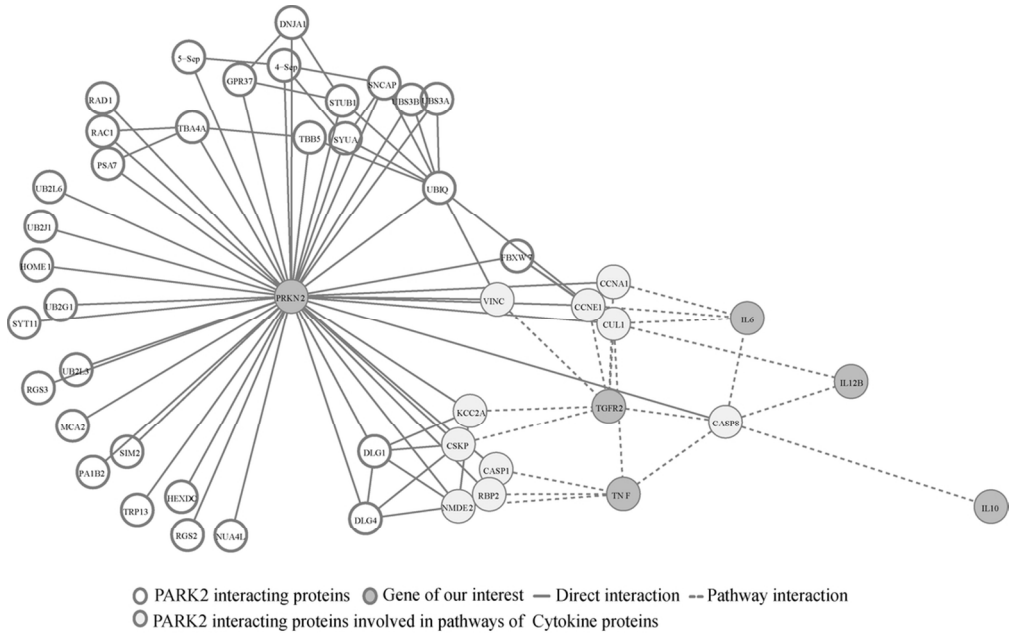

Figure 1
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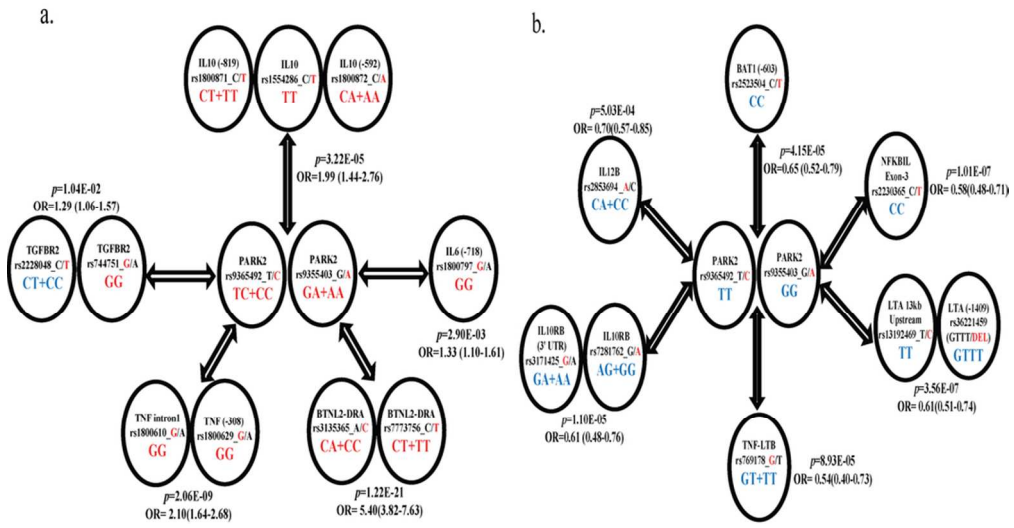

Figure S1
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Table S1: Pathway analysis of PARK2 interacting protein with Cytokines studied by us in Indian population groups

PARK2-interacting proteins	Uniport_ID of PARK2 interacting proteins	PATHWAYS involving PARK2 interacting proteins and Cytokine genes	Cytokine genes	Databases used for Pathways retrieval
CUL1	Q13616	Adherens junction	TGFR2	KEGG
VINC	P18206	Adherens junction	TGFR2	KEGG
CUL1	Q13616	Amyotrophic lateral sclerosis (ALS)	TNF	KEGG
NMDE2	Q13224	Amyotrophic lateral sclerosis (ALS)	TNF	KEGG
CUL1	Q13616	Angiopoietin receptor Tie2-mediated signaling	TNF	Nci-Nature
CASP8	Q14790	Apoptosis	TNF	KEGG
CASP8	Q14790	caspase cascade in apoptosis	TNF	BioCarta
CASP8	Q14790	Caspase cascade in apoptosis	TNF	Nci-Nature
CASP1	P29466	caspase cascade in apoptosis	TNF	BioCarta
CASP1	P29466	Caspase cascade in apoptosis	TNF	Nci-Nature
CASP1	P29466	Cellular roles of Anthrax toxin	TNF	Nci-Nature
CASP8	Q14790	ceramide signaling pathway	TNF	BioCarta
CASP8	Q14790	Ceramide signaling pathway	TNF	Nci-Nature
CASP8	Q14790	Chagas disease	IL10, TGFR2, TNF	KEGG
CUL1	Q13616	Fc epsilon RI signaling pathway	TNF	KEGG
CASP8	Q14790	hiv-1 nef: negative effector of fas and tnf	TNF	BioCarta
CASP8	Q14790	HIV-1 Nef: Negative effector of Fas and TNF-alpha	TNF	Nci-Nature
CUL1	Q13616	IL6-mediated signaling events	IL6	Nci-Nature
CUL1	Q13616	Integrins in angiogenesis	TGFR2	Nci-Nature
CASP8	Q14790	Integrins in angiogenesis	TGFR2	Nci-Nature
VINC	P18206	Integrins in angiogenesis	TGFR2	Nci-Nature
CUL1	Q13616	LPA receptor mediated events	IL6	Nci-Nature

CUL1	Q13616	MAPK signaling pathway	TGFR2, TNF	KEGG
CUL1	Q13616	Natural killer cell mediated cytotoxicity	TNF	KEGG
RBP2	P49792	Signaling events mediated by HDAC Class I	TNF	Nci-Nature
CSKP	O14936	Syndecan-1-mediated signaling events	TGFR2	Nci-Nature
KCC2A	Q9UQM7	TGF-beta receptor signaling	TGFR2	Nci-Nature
CUL1	Q13616	TGF-beta signaling pathway	TGFR2, TNF	KEGG
CASP8	Q14790	TNF receptor signaling pathway	TNF	Nci-Nature
CASP8	Q14790	TNF signaling	TNF	Reactome
CASP8	Q14790	tnfr1 signaling pathway	TNF	BioCarta
CUL1	Q13616	Toll-like receptor signaling pathway	IL6, TNF, IL12B	KEGG
CASP8	Q14790	Toll-like receptor signaling pathway	IL6, TNF, IL12B	KEGG