

Footprints of genetic susceptibility to pulmonary tuberculosis: Cytokine gene variants in north Indians

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Background & objectives: Tuberculosis is (TB) responsible for high morbidity and mortality worldwide. Cytokines play a major role in defense against *Mycobacterium tuberculosis* infection. Polymorphisms in the genes encoding the various pro- and anti-inflammatory cytokines have been associated with tuberculosis susceptibility. In this study we examined association of 25 sequence polymorphisms in six candidate cytokine genes namely *IFNG*, *TNFB*, *IL4*, *IL1RA*, *IL1B* and *IL12* and their related haplotypes with risk of developing pulmonary tuberculosis (PTB) among north Indians.

Methods: Pulmonary TB (n=110) patients and 215 healthy controls (HC) from north India were genotyped. Purified multiplex PCR products were subjected to mass spectrometry using Sequenom MassARRAY platform to generate the genotypes in a population-based case-control study.

Results: Using multiple corrections, significant overall risk against PTB was observed at seven loci which included variants in *IFNG* at rs1861493 and rs1861494; *IL1RA* at rs4252019, *IL4* variant rs2070874, *IL12* variants rs3212220, rs2853694 and *TNFB* variant rs1041981. Analysis of gene structure revealed two haplotype blocks formed by *IFNG* variants rs1861493 and rs1861494. The TA haplotype was significantly over-represented ($P=0.011$) in the cases showing a two-fold risk in the current population (Odds ratio=1.59 CI=1.101 to 2.297) and *TNFB* variants at rs2229094 and rs1041981 contributed to two haplotypes which were in strong linkage disequilibrium (LD) with AT haplotype showing a three-fold risk ($P=0.0011$, Odds ratio=3, CI=0.1939 to 0.7445) of developing PTB in north Indians.

Interpretation & conclusions: Our study showed six novel associations of cytokine gene variants with susceptibility to PTB in north Indians. Variants of *IFNG* and *TNFB* emerged as factors imposing a significant risk of developing PTB in north Indians apart from risk indicated by *IL1RA*, *IL4* and *IL12*.

Key words Cytokine gene variant - haplotype - *Mycobacterium tuberculosis* - pulmonary tuberculosis - single nucleotide polymorphisms

Tuberculosis (TB) causes significant morbidity and mortality throughout the world¹. The vast majority of individuals infected with *Mycobacterium tuberculosis* (up to 95%) remain healthy, probably because of mounting an effective immune response

against *M. tuberculosis*. In 1949, Haldane proposed that the maintenance of multiple genes that confer relative susceptibilities on the host to infectious diseases would be favoured by evolution. In support of this hypothesis, certain populations appear to be

at risk for both increased susceptibility to infection² and progressive clinical disease due to mycobacteria³. Several case-control studies have identified association between TB and candidate genes potentially involved in immune response to TB^{4,5}. A growing body of evidence supports a role of host genetic components in the development of tuberculosis. The observation of familial clustering of disease with higher concordance of tuberculosis disease in monozygotic versus dizygotic twins⁶, the ethnic clustering of tuberculosis disease with a higher prevalence of tuberculosis in individuals of recent African descent², as well as the demonstration of both common polymorphisms and rare mutations which confer susceptibility to mycobacterial species in humans⁷ point significantly in this direction. These studies suggest that unique environment and natural selective factors may be responsible for the development of ethnic-specific host genetic factors associated with TB.

The first step in innate host defense is cellular uptake of *M. tuberculosis*, which involves different cellular receptors and humoral factors. The subsequent inflammatory response is regulated by the production of pro- and anti-inflammatory cytokines and chemokines. Interferon-gamma (IFN- γ) one of the most important cytokines involved in macrophage activation, stimulating anti-tumour and anti-microbicidal activities as well as expression of MHC-II^{8,9}. Interleukin-4 (IL-4), an anti-inflammatory cytokine has been implicated to downregulate IFN- γ , and thus has a deleterious effect on TB patients¹⁰. It also promotes the induction of Th2 cells¹¹. IL-12, a heterodimeric pro-inflammatory cytokine produced by activated macrophages, monocytes, β -lymphocytes and dendritic cells is the principal Th1 response inducing cytokine¹¹. This cytokine is important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to intracellular pathogen. Like tumour necrosis factor-alpha (TNF- α), IL-1 β is mainly produced by monocytes, macrophages, and dendritic cells¹². In tuberculosis patients, IL-1 β is expressed in excess¹³ and at the site of disease¹⁴. Implicated mainly in tuberculosis pleurisy, a usually self-resolving type of primary tuberculosis, one may hypothesize that an increased IL-1 β /IL-1Ra ratio protects against a more severe form of tuberculosis.

TNF- β or lymphotoxin-alpha (LT α) is considered to be a proinflammatory cytokine and it is shown that secreted LT α is essential for the control of an intracellular bacterial infection¹⁵. Recently Allie *et al*¹⁶

suggested that LT α might not have a critical role in host defense to acute mycobacterial infection, independent of TNF, but certainly a contribution of LT α in the control of chronic *M. tuberculosis* infection is observed¹⁷.

Association studies from north India probing multiple loci across the spectrum of candidate cytokine genes are scanty. The present study, therefore, was aimed to bring in focus certain unexplored polymorphisms in the context of tuberculosis susceptibility in north Indian population. The role and importance of genetic background in tuberculosis has now become univocal with ethnicity playing a crucial role. Probing new loci relating to tuberculosis susceptibility could suggest novel approach in pharmacogenomics and therapy to combat this pathogen. Also it could provide an insight into predicting individual's genetic proneness to tuberculosis and of being future diagnostic tool for preventive therapy against tuberculosis.

Material & Methods

Study population: PTB patients above 18 yr of age (n=110) were enrolled randomly in the study between 2010-11 from Rajan Babu Institute of Pulmonary Medicine and Tuberculosis (RBIPMT), Kingsway Camp, New Delhi (India). The study was carried out in Department of Microbiology, V.P. Chest Institute, University of Delhi, Delhi. Enrolled patients were category I cases, clinically and radiologically (chest X-ray) diagnosed for pulmonary tuberculosis and confirmed by sputum microscopy and culture for *Mycobacterium* following the guidelines of Revised National TB Control Programme (RNCTP), Ministry of Health and Family Welfare, Government of India (<http://www.tbcindia.nic.in>). All patients were given free anti-tuberculosis drugs under DOTS (Directly Observed Treatment, short course) regimen of the Government of India. The mean age of PTB cases was 31.89 ± 2.6 yr while the ratio of male : female was 47:53.

Patients having any immunosuppressive presentation such as diabetes mellitus or HIV co-infection which are considered to be risk factors for tuberculosis development, and patients suspected to have extra-pulmonary tuberculosis along with pulmonary tuberculosis were excluded from the study. Structured questionnaires were used to document all other relevant information such as age, sex, ethnicity, socio-economic status, BCG vaccinations, and previous family history of tuberculosis. The healthy control (HC) group consisted of 215 randomly chosen

nonconsanguineous BCG vaccinated students and laboratory personnel from the various departments of the University of Delhi who were willing to participate in the study with no signs, symptoms or history of previous mycobacterial infection. For HC mean age was $29.31 \pm .82$ yr and the ratio of male : female was 43:57.

Analysis of population stratification: Serious effort was made to avoid any false-positives arising as a result of population stratification. The self reported ethnicity of each subject and his/her parents was carefully considered. In addition, the genotype data were subjected to EIGENSTRAT principal component analysis for population stratification correction as illustrated by Price *et al*¹⁸.

All individuals were briefed about the study and a signed informed consent was obtained from the patient or his or her guardians before sample collection. The study was approved by the ethics committee of Vallabhbhai Patel Chest Institute, University of Delhi, India.

DNA extraction: Three ml of venous blood was collected in BD vacutainers containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant and kept frozen until use. Genomic DNA was extracted from frozen whole blood using QiaAMP DNA kit (Qiagen, Germany). Extracted DNA was quantified by spectrophotometry, checked for purity and stored at -20°C until further analyses.

SNP selection and genotyping: Six candidate cytokine genes namely *IFNG*, *TNFB*, *IL4*, *IL1RA*, *IL1B* and *IL12B*, were selected owing to their suggested role in tuberculosis pathogenesis. All single nucleotide polymorphisms (SNPs) selected for genotyping were accessed from the public dbSNP (<http://www.ncbi.nih.gov>) and the HapMap (<http://www.hapmap.org/>). Most of the selected SNPs are from the intronic regions of the corresponding genes. We reasoned that not only the changes in the promoter but also of other unexplored regions of the gene may hamper its normal functioning leading to disease. The parameters taken into account while SNP selection were the frequency of <0.01 in dbSNP, reported allele frequency of at least 20 per cent in two world populations (from Hapmap), average spacing 1 kb but in closely spaced minor allele frequency was carefully considered. In addition, reported heterozygosity was considered in an effort to minimize selection of homozygous loci.

All SNPs were genotyped using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc., USA). Assays for all SNPs were designed using SpectroDESIGNER software (Sequenom Inc., USA). All SNPs were genotyped using the iPLEX assays (www.sequenom.com/ipllex). Briefly, as template, 5 ng of genomic DNA was used in a multiplex PCR reaction. The PCR product was further purified before the primer extension reaction to generate allele-specific base extension products. The base-extension products were detected in the MALDI-TOF mass spectrometer to determine genotypes.

Genetic and statistical analyses: Hardy-Weinberg equilibrium was calculated in both PTB cases and HC separately to ensure that the samples were within allelic population equilibrium by using Haploview v 4.2 (<http://www.broad.mit.edu/mpg/haploview/>). A stringent cut-off offered by the Haploview v 4.2 was used for further analysis (minimum genotype =75% and minimum minor allele frequency 0.0010). The samples and variations failing this test were not selected for further analysis. PLINK v 1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>) was used to test for multiple comparison and *P* value after Bonferroni corrections was considered significant. Haplotype block generation was performed using the algorithm by Gabriel *et al*¹⁹ implemented in the Haploview software which was also used for initial association testing. The statistical significance of *P* value of haplotypes was assessed by permutation analysis (N=10,000) with Haploview v 4.2.

Genetic association testing was done using a 2 x 2 contingency table. Odds ratio, two tailed *P* value was calculated for alleles. 2 x 2 Computations were done using GraphPad Prism (version 5.00 for Windows, Graph Pad Software, San Diego California, USA; www.graphpad.com). Two-tailed *P*<0.05 was considered statistically significant.

Results

Table I shows the location and characteristics of the SNPs included in the study and Table II shows the associations after multiple corrections carried out using PLINK (<http://pngu.mgh.harvard.edu/purcell/plink/>) which were found to be associated with susceptibility to PTB in north Indians in this study.

Population stratification correction: To access any underlying structure in the study population that could

Table I. Location and base-pair positions of single nucleotide polymorphisms (SNPs) of various cytokine genes passing the exclusion criteria and minor allele frequency (MAF) in controls

Gene name	db SNP ^a rsID	Base change	Chromosome position	Location	MAF controls	References
<i>IFNG</i>	rs1861493	A/G	68551196	Intron 4	0.13	New; this study
	rs1861494	C/T	68551409	Intron 4	0.14	New; this study
<i>IL4</i>	rs2070874	C/T	132009710	5'- UTR	0.25	Moller <i>et al</i> ,2010 ²⁰
<i>TNFB</i>	rs1041981	A/C	31540784	Exon 4	0.26	New; this study
<i>IL12</i>	rs2853694	A/C	158749088	Intron 4	0.5	New; this study
	rs3212220	G/T	158754195	Intron 4	0.28	Moller <i>et al</i> ,2010 ²⁰
<i>IL1RA</i>	rs4252019	C/T	113889119	Intron 5	0.05	New; this study

^a db SNP, the SNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP>)

confound the apparent genetic association population stratification correction was carried out using Eigenstrat Principal Component analysis method as illustrated by Price *et al*¹⁸. The method models ancestry difference between cases and controls and any other compared group based on the supplied genotype data. Our cases and controls formed a homogenous group devoid of any stratification. According to Indian Genome Variation Consortium (IGVC)²⁰ north Indians fall into Indo-European lineage. Our cases and controls matched with supplied marker data of Indo-European ancestry thereby ruling out completely any underlying structure in the population.

Allelic association of cytokine SNPs and the risk of pulmonary tuberculosis: Among the 25 studied SNPs, from six candidate cytokine genes the variants of *IFNG*, *IL1RA*, *IL4*, *IL12* and *TNFB* were found to be associated with susceptibility to PTB in north Indians. All studied variants passing the exclusion criteria were in Hardy-Weinberg equilibrium in both cases and

controls. Allelic association when probed in variants passing the exclusion criteria yielded six loci showing high risk for PTB susceptibility.

IFNG polymorphism and PTB susceptibility: After adjusting for multiple testing corrections the *IFNG* intronic variants at rs1861493 [$\chi^2 = 12.089$, $P_{\text{bonferroni}} = 0.006593$, odds ratio (95%CI) = 3.8 (1.7 - 8.6)] and rs1861494 ($\chi^2 = 10.466$, $P_{\text{bonferroni}} = 0.01581$, odds ratio (95%CI) = 3.0 (1.5 - 5.6)) showed a significant risk of developing pulmonary tuberculosis in north Indians with over-representation of the associated A and T alleles among PTB patients, respectively. Investigation of the gene structure and linkage disequilibrium pattern showed haplotypes formed by *IFNG* variants rs1861493 and rs1861494 which were in high linkage disequilibrium (LD) (Fig.). Three combinations of haplotype were seen namely TC, CC and TA, of which TA haplotype was over-represented in the cases and imposed a two-fold risk of developing pulmonary tuberculosis in north Indians (Table III).

Table II. Allelic associations in after adjustment for multiple testing

Gene	db SNP ^a rsID	Case (n=110), control (n=215) frequencies	Odds ratio (95%CI)	Chi square	P value*	P _{bonferroni} [#]
<i>IFNG</i>	rs1861493	0.962, 0.869	3.8 (1.7 - 8.6)	12.089	5.00E-04	0.00659
	rs1861494	0.946, 0.859	3.0 (1.5 - 5.6)	10.466	0.0012	0.01581
<i>IL4</i>	rs2070874	0.387, 0.255	1.8 (1.3 - 2.6)	10.708	0.0011	0.01387
<i>TNFB</i>	rs1041981	0.356, 0.238	1.7 (1.2 - 2.6)	8.649	0.0033	0.03618
<i>IL12</i>	rs2853694	0.607, 0.478	1.6 (1.2 - 2.4)	8.854	0.0029	0.0399
	rs3212220	0.419, 0.263	2.0 (1.4 - 2.9)	14.572	1.00E-04	0.00175
<i>IL1RA</i>	rs4252019	1.000, 0.935	14.0 (1.8 - 103.5)	13.643	2.00E-04	0.00287

SNP, single nucleotide polymorphism; *unadjusted P- value; #P value after bonferroni multiple testing correction; ^adb SNP, the SNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP>); P<0.05 was considered significant

Table III. Heplotype blocks and frequencies

Blocks	Haplotype frequency	Case (n=110), control (n=215) frequencies	Chi square	Permutations P value #	Odds ratio	(95% CI)
Block 1						
TC	0.41	0.36, 0.43	2.82	0.093	0.75	(0.53 - 1.1)
CC	0.32	0.29, 0.33	0.86	0.353	0.85	(0.59 - 1.2)
TA	0.27	0.34, 0.24	6.46	0.04	1.59	(1.1 - 2.3)
Block 2						
AT	0.88	0.95, 0.86	10.66	0.005	2.9	(1.5 - 5.6)
GC	0.10	0.05, 0.13	8.85	0.017	0.38	(0.2 - 0.7)

#P value after performing permutation (n=10,000); P<0.05 was considered significant

IL4 polymorphism and PTB susceptibility: *IL4* variant rs2070874 [$\chi^2=10.708$, $P_{\text{bonferroni}} = 0.01387$, odds ratio (95%CI) = 1.8 (1.3 - 2.6)] showed a two-fold risk by T allele in north Indians. The other studied *IL-4* variant rs2243270 passing the exclusion criteria did not show any association towards susceptibility to pulmonary tuberculosis in this population.

IL1RA polymorphism and PTB susceptibility: The significantly associated locus of *IL1RA* included intronic variant at rs4252019 [$\chi^2=13.643$, $P_{\text{bonferroni}} = 0.00287$, Odds ratio (95%CI) = 14.0 (1.8 - 103.5)] showing a 14-fold risk. Other variant such as rs315919 and rs380092 did not show any association towards susceptibility to pulmonary tuberculosis in this population.

IL12 polymorphism and PTB susceptibility: *IL12* variants rs3212220 [$\chi^2=14.572$, $P_{\text{bonferroni}} = 0.00175$, Odds ratio (95%CI) = 2.0 (1.4 - 2.9)] and rs2853694 [$\chi^2=8.854$, $P_{\text{bonferroni}} = 0.0399$, odds ratio (95%CI) = 1.6 (1.2 - 2.4)] showed a two-fold risk associated with T and A alleles, respectively.

IL1B polymorphism and PTB susceptibility: The selected *IL1B* variants did not show any direct influence on PTB susceptibility in north Indians.

TNFB polymorphism and PTB susceptibility: *TNFB* variants at rs1041981 [$\chi^2=8.649$, $P_{\text{bonferroni}} = 0.03618$, Odds ratio (95%CI) = 1.7 (1.2 - 2.6)] a synonymous change showed a two-fold risk of association for PTB in north Indians. Interestingly rs1041981 contributed to a haplotype block with rs2229094 confirming the importance of this locus in risk of developing PTB in north Indians. The two haplotypes observed were AT and GC of which AT was over-represented in PTB cases and imposed a three-fold risk of developing PTB in north Indians.

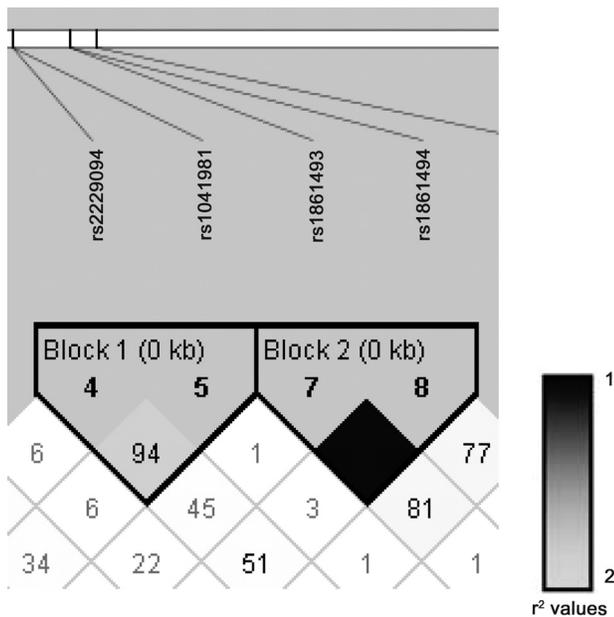


Fig. Linkage disequilibrium (LD) plot and haplotype structure of cytokine gene variants in PTB cases. D' values are displayed within each diamond, missing values indicate D' = 100%. Colour scheme gradient indicates r² values. Length of each block, in kilobases (kb), is shown in brackets.

Discussion

The host genetic bias contributing to susceptibility and progression of pulmonary tuberculosis might

involve interactions between multiple alleles located on different genes and chromosomes²¹. In order to overcome this drawback we planned selection of different cytokine gene and multiple loci to cover a wide spectrum of immune response associated cytokines.

Case-control studies involving carefully chosen locus across ethnicities are valiant means of identifying novel associations pertaining to disease susceptibility. Association that arises may be a result of the polymorphism in question being functional or it being in linkage disequilibrium with another functional allele or a result of confounding association due to population stratification. To overcome such false positives, we carefully considered the self reported ethnicity of the study groups and further checked for any genetic heterogeneity in our data by Eigenstrat principal component analysis illustrated by Price *et al*¹⁸ and found that the present data were free from any underlying population structure. Thus, this uniform data represent north Indian population for association analysis.

The IFN- γ being a crucial cytokine in immunopathogenesis of TB has been subject to several polymorphisms studies for pulmonary tuberculosis susceptibility. The locus probed here namely rs1861494 has not been studied in susceptibility to PTB but extensively studied in many other diseases such as leprosy²² and asthma²³. Kumar *et al*²⁴ found an association of this locus with susceptibility to asthma in Indians and could identify a haplotype. They also showed that alleles of rs1861494 A/G have differential affinity to bind to putative nuclear factor. In the present study, we found significant risk for the locus in susceptibility to PTB. The other probed locus rs1861493 has been studied in idiopathic inflammatory myopathy²⁴ and asthma²³ but not in pulmonary tuberculosis. We also identified a risk haplotype contributed by rs1861493 and rs1861494 emphasizing the importance of the above mentioned loci as risk factors for developing pulmonary tuberculosis in north Indians.

IL4 locus rs2070874 has been an important locus of investigation in various diseases including asthma and rheumatoid arthritis²⁵. Its role in TB was reported not to be significant in Iranian pulmonary TB patients²⁶ and recently in South Africans TB patients also the locus did not show any association²⁷. In the present study this locus showed a two-fold risk in the north Indian population.

IL1RA locus rs4252019 has shown significant risk of development of pulmonary TB in north Indians. The variant rs4252019 has been shown to be associated with prostate cancer risk²⁸ but not pulmonary tuberculosis. Interestingly, the variant showed a 14-fold risk of developing PTB in the population studied here and emerged as a major locus to look out for in further studies.

IL12 variants rs3212220 and rs2853694 showed a significant risk associated with development of PTB in north Indians. The variant rs3212220 has been shown to contribute to a haplotype by Moller *et al*²⁰. We have also predicted its importance in our previous study²⁹. Based on the analysis of serum IL-12 level, we demonstrated that for *IL12* variant rs3212220 TT genotype among active PTB cases showed significantly higher serum IL-12 level when compared to either GT or GG. The present study revealed T allele to be a risk allele in the present population. Similarly, rs2853694 a novel variant in the context of developing tuberculosis²⁹ was predicted to be of importance and was validated in the present study. For rs2853694 among active PTB cases AA genotype showed a trend towards higher serum IL-12 level in contrast to a reverse trend observed in HC where AA accounted for low serum IL-12²⁹. The present study showed A allele at rs2853694 to be a risk allele for the north Indian population in the context of PTB susceptibility. An interesting observation was that both the higher serum cytokine producers *i.e.* TT genotype for rs3212220 and AA genotype for rs2853694 emerged as respective risk alleles T and A for this population, indicating that overproduction of IL-12 by these individuals might be interfering with the cytokine homeostasis and thus affecting the immune function of the cytokine in these individuals making them prone to infection. Our observation was further supported by the work of Leandro *et al*³⁰, who indicated that role of IL-12 as potent inducer of IFN- γ lied in its efficacy at low concentrations. In the present study it is observed that the PTB patients with IL12 risk allele genotypes are not efficient inducers of IFN- γ which in turn interferes with the protective immunity in these individuals, whereas a low profile of IL-12 in HC elicits an effective and optimal immune response rendering these individuals healthy.

TNFB though not usually considered for PTB association studies, was taken up in the current study because of its role in control of intracellular bacterial infection¹⁵. The variant rs1041981 emerged as a

significant risk locus for PTB susceptibility in north Indians. The variant also contributed to a haplotype with rs2229094 and reinstated the role of *TNFB* polymorphisms in PTB.

Overall, five of the loci namely rs1861493 and rs1861494 (*IFNG*), rs4252019 (*IL1RA*) rs1041981 (*TNFB*) and rs2853694 (*IL12*) studied in patients of pulmonary tuberculosis showed a significant risk towards susceptibility to pulmonary tuberculosis in north Indians. We also report here the significant risk imposed by *IL4* variant rs2070874 in the active PTB patients. Six new associations and three new associated haplotypes contributing to the spectrum of cytokine gene polymorphisms and risk of developing tuberculosis in general and north Indians in particular, were detected.

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