

Association of *IL4RA* and *IL-8* polymorphism in predicting susceptibility to tuberculosis

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Abstract

Mycobacterium tuberculosis causes an infectious bacterial disease frequently affecting the lungs. With two fatalities from tuberculosis (TB) occurring every three minutes, India has the highest disease burden. The etiology of TB has been linked to *IL-8* and *IL-4RA*. Thus, the impact of the *IL4RAQ576R* and *IL8* gene polymorphisms on TB susceptibility was assessed. 301 healthy and 301 TB patients participated in a cross-sectional study. Polymerase chain reaction-restriction fragment length polymorphism was performed to identify the genotype of the *IL4RAQ576R* and *IL-8 + 781C/T* gene polymorphism. The odds ratio and 95% confidence intervals were calculated using logistic regression to evaluate the risk of TB with *IL4RAQ576R* and *IL-8 + 781 C/T* polymorphism. A significant association was found between *IL-4RA* ($p=0.04$) and *IL-8 + 781 C/T* ($p=0.03$) in TB. Further, when clinical symptoms were compared with both polymorphisms, two of them, *i.e.*, cough in *IL-4RAQ576R* ($p=0.04$) and breathlessness ($p=0.01$) in *IL-8 + 781 C/T*, showed a significant association. Moreover, different combinations of the single nucleotide polymorphisms (SNPs) were made, and the 3-risk allele shows a significant protective role ($p=0.02$). There is considerable evidence showing that *M. tuberculosis* causes TB, an infectious disease that is genetically predisposed. The results of our study also showed that *IL-4 RA Q576R* and *IL-8 + 781 C/T* played a significant protective function against TB, confirming the claim mentioned earlier. However, only the cough in *IL-4RAQ576R* and the dyspnea in *IL-8 + 781 C/T* exhibited a significant co-relation in TB patients when symptoms were examined. Additionally, the combined effects of the two SNPs were investigated, and it was discovered that the 3-risk allele has a strong association with TB. Therefore, the polymorphisms mentioned earlier, which may also be influenced by ethnicity, may significantly impact the chance of developing TB.

Introduction

Tuberculosis (TB) is an infectious disorder caused by *Mycobacterium tuberculosis* (Mtb) and recognized as a significant cause of single-infection source-associated mortality worldwide [1]. Several factors can increase the infection risk and disease progression of TB, including malnutrition, smoking, diabetes, alcohol use, socioeconomic status, and environmental pollution [2]. Moreover, many studies have shown diverse genetic factors' vital role in host vulnerability to TB [3]. A growing number of strains are resistant to one or more anti-TB drugs, constituting the leading cause of the decreasing efficiency of chemotherapy, increasing the number of patients with destructive forms, the frequency of significant residual post-TB alterations, and the incidence of TB relapses. Interleukin

(IL)-8, an inflammatory cytokine of the chemokine superfamily with functional and structural correlation, was first discovered in 1987 [4]. The *IL-8* gene, which spans an area of about 2.75 Mb on chromosome 4, is located in the region q13.3 and has four exons, three introns, and the proximal promoter region. It encodes a protein with 99 amino acid residues and a transcript length of 1705 base pairs [5]. The effects of IL-8 on human TB have become a particular research hotspot globally. Pathological and clinical observations have revealed prominent elevations of IL-8 levels in cerebrospinal fluid, bronchoalveolar lavage fluid, and tuberculous pleural exudate [6]. *In vivo* studies have shown that IL-8 is vital for a normal immune response to TB infection and that anti-IL-8 inhibits granuloma formation. *IL-8* gene regulation in monocytes and macrophages is complex, and stimulus- and cell-type dependent. In most cells, it is regulated primarily at the gene transcriptional by transcriptional regulators CCAAT/enhancer binding protein β (C/EBP β), NF-kappa β , AP-1 and Oct-1 all having functional binding sites in the *IL-8* promoter [7]. The *IL-8* gene has three frequent polymorphisms: 251 A/T, +396 G/T, and +781 C/T (rs2227306). Of these, rs2227306 located on intron 1 may impact the transcriptional process because C/EBP bound preferentially in the presence of rs2227306T variation in respiratory epithelial cells [7].

IL-4R α is a component of both the IL-4 and the IL-13 receptor complexes, which explains the similarity of the biological effect of IL-4 and IL-13 [7]. A moderate shielding effect of the variant allele at several closely related single nucleotide polymorphisms (SNPs) has been documented by a polymorphism in IL-4R α . rs1801275 (A/G) of the *IL4RA* gene in chromosome 16 (exon 12, g.54150A>G) generates a missense change (p. Gln576Arg) which effect its binding with IL-4. Thus, ending up with a modulated IL-4 pathway [8]. The role of T helper (Th) 2 cell-mediated immunity manifested by IL-4 and IL-13 production in the pathogenesis and susceptibility remains a subject of discussion [9]. In TB, increased production of IL-4 and IL-13 is associated with lung damage, as some studies showed an increase in cavity size, indicating that signals are mediated through the common IL-4R α [10].

According to ALlele FREquency Database, where details on each population and sample are described, globally, rs2227306 has shown a frequency of 0.63 for the C allele and 0.36 for the T allele, whereas, in the case of rs1801275, the A allele has a frequency of 0.77 and the G allele has 0.22. Compared to the European population, frequency change has been seen for rs 2227306 (C=0.58, T=0.41), whereas no such change was seen in rs1801275 (A=0.79, G=0.20). In the Asian population, rs2227306 has a frequency of 0.578 and 0.422 for C and T, respectively, while 0.86 and 0.13 for C and T, respectively, in an African population. Whereas for rs1801275, it is 0.33 and 0.82 for A and 0.66, 0.16 for G in African and Asian populations, respectively.

Hence, our study aimed to elucidate the role of the *IL4RA* gene and *IL-8* +781 C/T polymorphism as a genetic marker for the risk of TB in the North Indian population. This study is the first to discover a relationship between *IL4RA* and *IL-8* +781 C/T polymorphisms and various clinical parameters of TB patients in the same population.

Materials and Methods

Study design

A cross-sectional study was conducted. The institutional ethical committee approved the study. 301 people were enrolled at the Department of Pulmonary Medicine, Chest and TB Hospital,

Government Medical College, Patiala (including 301 cases of chronic obstructive pulmonary disease and 301 healthy individuals). According to the TB diagnosis criteria, 301 cases and 301 healthy individuals were recruited. Blood samples were obtained and stored in EDTA-coated vacutainers at 20°C until DNA was isolated.

DNA extraction and genotyping

Whole blood samples from TB patients and healthy individuals were used to isolate genomic DNA using Barlett and White's method [11], which involved conventional protein K digestion, phenol/chloroform extraction, and ethanol precipitation. The presence of genomic DNA was examined on 0.8% agarose gel. The *IL-4RA* and *IL-8* polymorphisms were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis. The target sequence was amplified by PCR using primers under a single set of reaction conditions. The PCR mixture of 15 μ L comprised of 1X PCR buffer, 10 X Bovine Serum Albumin, 0.5 μ M forward primer, 0.5 μ M reverse primer of *IL4RAQ576R* and *IL-8* C/T polymorphism, 0.2 μ M dNTPs and 3 U/ μ L of Taq polymerase and 300ng of DNA. The primers used to amplify *IL-8* 781 C/T variants were FP: 5'-CTCAACTCTTTATATAGGAATT -3&; RP: 5'-GATTGATTTTATCAACAGGCA -3 &; and for *IL-4RA* variants were: FP: 5'- GCCTTGTAACCAGCCTCTCCT - 3& RP: 5'-5-GCCCCACCAGTGGCTACC-3. The PCR was run under the following conditions: denaturation step – 5 minutes at 95°C and 30 seconds at 94°C, annealing step – 45 seconds at 57°C (*IL-4RA*) and 42°C (*IL-8*), extension step – 29 cycles for 30 seconds each at 72°C and the final extension step – 5 minutes at 72°C. Msp I (*IL-4RA*) and EcoRI (*IL-8* +781 C/T) enzyme was used to perform the restriction digestion of the amplicon and were incubated at 37°C. Following digestion, the PCR products were separated on a 6% polyacrylamide gel, and the digested bands were identified using EtBr staining. The restricted digested pattern of *IL4RAQ576R* was as follows: AA-wild type (107 bp and 16 bp), AG-heterozygous (107bp, 89bp, 18 bp and 16bp) and GG-mutant alleles (89bp and 18bp) (Figure 1). For *IL-8*, the mutant allele produced a single band of 203 bp, the wild allele produced two bands of 184 & 19 bp, as shown in Figure 2, and the heterozygous allele produced three bands of 203, 184 & 19 bp.

Statistics

The distribution of demographic attributes between cases and healthy individuals was compared using the Student *t*-test for continuous variables and the χ^2 test for categorical data. In both cases and healthy individuals, the genotype frequencies of the

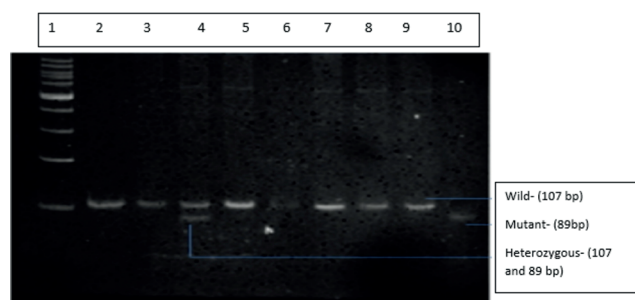


Figure 1. 6% polyacrylamide gel of digested polymerase chain reaction for the detection of genotypes of *IL-4RA* 576R polymorphism.

IL4RAQ576R and *IL-8 781 C/T* gene polymorphism were determined using the χ^2 test and the Hardy-Weinberg equilibrium (HWE) theory ($p^2+2pq+q^2=1$), where p was the frequency of the wild-type gene, and q was the number of variant alleles. To determine whether there was a significant difference in allele and genotype frequencies between cases and healthy individuals, Pearson's χ^2 test was used. The adjusted odds ratios (AORs) and 95% confidence intervals (CIs) for the risk of TB associated with the *IL4RAQ576R* and *IL-8 781 C/T* polymorphism were calculated using logistic regression analysis with correction for potential parameters (age, smoking, and gender as nominal variables) (CIs). A p-value <0.05 shall be considered to be statistically significant. For the statistical analysis, medical version 9.3.6.0 (Medcalc Software, Ostend, Belgium).

Results

Demographic and clinical characteristics among tuberculosis patients and healthy individuals

The sample population in the current study has been characterized by various demographic (*i.e.*, age, gender, smoking status) and clinical (*i.e.*, cough, breathlessness, fever, appetite, weight, chest pain, night sweats, hemoptysis) parameters as tabulated in Table 1. Briefly, 301 subjects for both cases and controls were recruited. Of 301 subjects, 183 were male, and 118 were females for both controls and cases, with the mean age at 34.25 ± 10.39 for cases and 35.7 ± 9.3 for controls; hence there was no significant difference between the mean ages of both cases and controls ($p=0.07$). No significant differences between the distribution of males and females in the case and controls were found. The number of smokers and non-smokers was

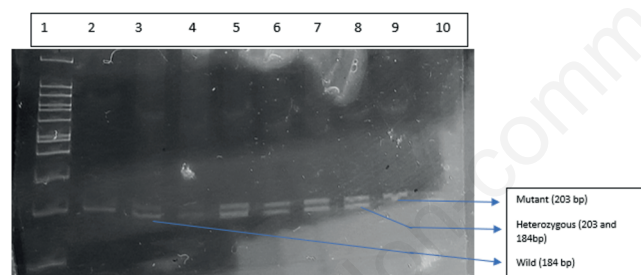


Figure 2. 6% polyacrylamide gel of digested polymerase chain reaction for the detection of genotypes of *IL-8 781C/T* polymorphism.

compared between the two study groups. The case group comprised 19.9% smokers, whereas controls had 21.3% smokers; no significant differences were observed in the proportion of smokers and non-smokers in our study.

Overall distribution and association of *IL4RAQ576R* polymorphism with tuberculosis risk

This study evaluated the relationship between *IL4RAQ576R* polymorphism and susceptibility to TB. The allelic and genotypic distribution of *IL4RA* polymorphism, *i.e.*, Gln⁵⁷⁶Arg, is shown in Table 2. No significant departure was observed from HWE for the *IL-4RA* genotype among cases and controls, signifying no sample bias. Statistical analysis revealed that TB patients had a lower representation of wild-type genotype (Gln/Gln), homozygous variant type (Arg/Arg) than controls (Gln/Gln: 57.48% versus 56.47%, and Arg/Arg: 3.32% versus 0.66%). In contrast, as shown in Table 2, heterozygous (Gln/Arg) was over-represented in cases compared to control (42.85% versus 39.20%). Therefore, no significant difference was observed for genotypic distribution amongst cases and controls ($\chi^2=5.849$, $df=2$, $p=0.067$). The minor allele frequency (MAF) for controls (0.23) was slightly higher as compared to cases (0.22). Table 2 further shows the association of TB risk with *IL-4RA* polymorphism. AORs and 95% CI were calculated using logistic regression analysis. To further explore the correlations between the *IL-4RA* variant and TB risk, three different genetic models (codominant, dominant, and recessive) were studied. The wild genotype was considered the reference. In the codominant model, 39.20% of healthy individuals and 42.85% of cases had the heterozygous (AG) genotype. When compared to the wild-type (AA) genotype, there was no real relationship between the heterozygous (AG) genotype and risk (OR=1.11, 95% CI=0.80-1.54, $p=0.52$). No significant association was discovered, even after adjusting for age, gender, and smoking status (AOR=1.10, 95% CI=0.80-1.00, $p=0.55$). When compared to the wild genotype, the mutant (GG) genotype indicates a marginally significant reduction in the risk of developing TB (0.66% in TB patients and 3.32% in healthy individuals) (OR=0.20, 95% CI=0.04-0.94, $p=0.04$). The dominant model (AA versus AG+GG) did not show any relationship for TB susceptibility, as shown in Table 2. Conversely, in the recessive model, the mutant genotype has a decreased effect, showing a protective effect towards TB.

Association of *IL4RAQ576R* polymorphism and clinical symptoms for tuberculosis

Based on clinical symptoms such as coughing, shortness of breath, lack of appetite, weight loss, hemoptysis, chest pain, fever,

Table 1. Demographic characteristics among cases and controls.

Variable	Total (n)	Cases, n (%)	Controls, n (%)	p*
Age (years)	301			
Mean±SD		34.25±10.39	35.7±9.3	0.07
Range		18-50	15-50	
Gender	301			
Male		183 (60.8)	183 (60.8)	
Female		118 (39.2)	118 (39.2)	1.00
Smoking status	301			
Smokers		60 (19.9)	64 (21.3)	
Non-smokers		241 (80.1)	237 (78.7)	0.68

SD, standard deviation; *p-values were derived from Pearson χ^2 test except for age and pack-years; Student's *t*-test was used for age and pack-years; all p-values are two-sided; $p<0.05$ was considered statistically significant.

and night sweats, we also evaluated the association between *IL4RAQ576R* polymorphism and TB risk. Three models, *i.e.*, codominant, dominant, and recessive models, were used and then adjusted with covariates like age, sex, and smoking. All the clinical symptoms were divided into two groups, whether the subject exhibited that symptom or not. For cough, in the codominant model, the mutant (GG) genotype was observed in 0.7% of patients having cough, and no mutant was observed in patients without cough. When compared to the wild-type genotype (AA), the heterozygous (GG) genotype was found to be associated with cough in TB patients, as shown in Table 3 (OR=2.36, 95% CI=1.02-5.48, p=0.04). There was a 2.36-fold increase in cough in TB patients even when adjusted for cough, age, and smoking (AOR=2.27, 95%CI=0.97-5.33, p=0.04). Furthermore, by combining the two risk genotypes, *i.e.*, heterozygous and mutant genotype (AG+GG) for the dominant model, we observed a 2.40-fold increased probability of cough in TB patients. Within the TB patients, breathlessness, weight loss, and appetite loss were seen in only 23.9%, 68.77%, and 69.76% of patients. When the codominant and dominant model was applied to the group of TB patients, no significant results were seen, even when adjusted with covariates like age, gender, and smoking. However, TB patients having heterozygous genotypes show a protective effect against breath-

lessness (AOR=0.87, 95%CI=0.51-1.51, p=0.64), loss of appetite (AOR=0.94, 95%CI=0.57-1.55, p=0.79) and weight loss (AOR=0.78, 95%CI=0.47-1.28, p=0.33). Further, an association between *IL-4RA* and night sweats and chest pain was evaluated. When we compared the two using an unconditional regression model, we discovered that the *IL-4RA* genotype was not associated with the codominant model. When heterozygous and mutant genotypes were pooled, the dominant model also demonstrated a protective effect against night sweats and chest pain (AOR=0.87, 95%CI=0.43-1.77, p=0.69) (AOR=0.87, 95%CI=0.43-1.77, p=0.69).

Overall distribution and association of *IL-8* polymorphism with tuberculosis risk

For this study, an association between *IL-8* polymorphism and TB was evaluated. The allelic and genotypic distribution of *IL-8 C/T* polymorphism is shown in Table 4. No significant departure was observed from HWE for *IL-8 C/T* genotype among cases and controls, signifying no sample bias. Statistical analysis revealed that TB patients had a higher representation of the wild-type genotype (C/C) than controls (C/C: 49.5% versus 41.8%). In contrast, heterozygous

Table 2. Genotypic and allelic distribution of *IL-4RA* genetic variant and its association with risk of tuberculosis.

Polymorphism <i>IL4RAQ576R</i>	Controls		Cases			
	n (%) n=301	n (%) n=301	OR (95% CI)	p [#] (95% CI)	AOR	p [§]
Codominant						
AA	173 (57.48)	170 (56.47)	1.00 (Reference)	-	1.00 (Reference)	-
AG	118 (39.20)	129 (42.85)	1.11 (0.80-1.54)	0.52	1.10 (0.80-1.00)	0.55
GG	10 (3.32)	2 (0.66)	0.20 (0.04-0.94)	0.04	0.20 (0.04-0.94)	0.04
Gln (allele)	464	469				
Arg (allele)	138	133				
MAF	0.23	0.22 $\chi^2=5.849$, df=2, p=0.067				
Dominant	n (%) n=301	n (%) n=301	OR (95% CI)	p [#]	AOR (95% CI)	p [§]
AA	173 (57.48)	170 (56.4)	1.00 (Reference)	-	1.00 (Reference)	-
AG+GG	128 (42.52)	131 (43.5)	1.04 (0.75-1.43)	0.80	1.03 (0.75-1.43)	0.83
Recessive	n (%) n=301	n (%) n=301	OR* (95% CI)	p [#]	AOR* (95% CI)	p [§]
AA+AG	291 (96.68)	299 (99.3)	1.00 (Reference)	-	1.00 (Reference)	-
GG	10 (3.32)	2 (0.66)	0.19 (0.042-0.90)	0.04	0.19 (0.04-0.90)	0.03

OR, odds ratio; 95% CI, 95% confidence intervals; AOR, adjusted odds ratio; MAF, minor allele frequency; #corresponding p-values were calculated by logistic regression analysis; §p-value after being adjusted.

Table 3. Association of *IL-4RA* and cough for tuberculosis.

Polymorphism <i>IL4RAQ576R</i>	With cough	Without cough	Cases			
	n (%) n=270	n (%) n=31	OR (95% CI)	p [#]	AOR (95% CI)	p [§]
Codominant						
AA	147(54.44)	8 (25.80)	1.00 (Reference)	-	1.00 (Reference)	-
AG	121 (44.81)	23 (74.19)	2.36 (1.02-5.48)	0.04	2.27 (0.97-5.33)	0.04
Dominant	n (%) n=270	n (%) n=31	OR (95% CI)	p [#]	AOR (95% CI)	p [§]
AA	147 (54.44)	23 (74.19)	1.00 (Reference)	-	1.00 (Reference)	-
AG+GG	123 (45.55)	8 (25.80)	2.40 (1.04-5.57)	0.04	2.30 (0.98-5.40)	0.04

OR, odds ratio; 95% CI, 95% confidence intervals; AOR, adjusted odds ratio; #corresponding p-values were calculated by logistic regression analysis; §p-value after being adjusted.

(C/T) and mutant (T/T) were over-represented in controls as compared to cases (C/T: 49.5% versus 45.5%, T/T: 8.6% versus 4.9%). Therefore, no significant difference was observed for genotypic distribution amongst cases and controls ($\chi^2=5.849$, $df=2$, $p=0.067$). The MAF for controls (0.33) was higher as compared to cases (0.27). When the codominant model was applied, the heterozygous (CT) genotype was found in 45.5% of TB patients and 49.5% of healthy individuals. No significant association was found between the heterozygous (CT) genotype and TB risk (OR=0.77, 95% CI=0.56-1.08, $p=0.14$) when compared to the wild-type (CC) genotype. Even when it was adjusted with covariates such as age, gender, and smoking status, no substantial relationship was found (AOR=0.78, $p=0.16$, 95% CI=0.56-1.10). The mutant (TT) genotype was observed in 4.96% of TB patients and 8.6% of healthy individuals. But compared with the mutant genotype, the T allele exhibits a protective effect towards TB. Similarly, when dominant (OR=0.73, $p=0.06$, 95% CI=0.53-1.01) and recessive (OR=0.74, $p=0.06$, 95% CI=0.54-1.02) models were applied, the mutant genotype had a

trend exhibiting protective effects towards TB even when adjusted with age, gender, and smoking.

Association of IL-8 781 C/T polymorphism and tuberculosis risk based on clinical symptoms

Our study assessed the association between *IL-8* C/T polymorphism and TB risk based on clinical symptoms like coughing, shortness of breath, lack of appetite, weight loss, hemoptysis, chest pain, fever, and night sweats. When all three models, *i.e.*, codominant, dominant, and recessive models, were used and then adjusted with covariates like age, sex, and smoking, no significant association was found in all the symptoms except breathlessness, which, when compared to wild-type genotype (CC) in the codominant model as shown in Table 5 (OR=1.35, 95% CI=0.78-2.39, $p=0.27$). Although the mutant (TT) genotype showed the risk of TB susceptibility, the association was significant (OR=0.22, 95% CI=0.06-0.75, $p=0.01$). Other clinical symptoms did not show any significant results, but TB

Table 4. Genotypic and allelic distribution of *IL-8* genetic variant and its association with risk of tuberculosis.

Polymorphism <i>IL-8</i>	Controls		Cases			
	n (%)	n (%)	OR (95% CI)	p [#]	AOR (95% CI)	p [§]
Codominant	n=301	n=301				
CC	126 (41.8)	149 (49.5)	1.00 (Reference)	-	1.00 (Reference)	-
CT	149 (49.5)	137 (45.5)	0.77 (0.56-1.08)	0.14	0.78 (0.56-1.10)	0.16
TT	26 (8.6)	15 (4.9)	0.49 (0.24-0.96)	0.03	0.50 (0.25-0.99)	0.04
C (allele)	401	435				
T (allele)	201	167				
MAF	0.33	0.27	$\chi^2=5.849$, $df=2$, $p=0.067$			
Dominant	n=301	n=301				
CC	126 (41.8)	149 (49.5)	1.00 (Reference)	-	1.00 (Reference)	-
CT+TT	175 (58.1)	152 (50.4)	0.73 (0.53-1.01)	0.06	0.74 (0.54-1.02)	0.06
Recessive	n=301	n=301				
CC+CT	275 (91.3)	286 (95.01)	1.00 (Reference)	-	1.00 (Reference)	-
TT	26 (3.32)	15 (3.96)	0.55 (0.28-1.06)	0.07	0.56 (0.28-1.08)	0.06

OR, odds ratio; 95% CI, 95% confidence intervals; AOR, adjusted odds ratio; MAF, minor allele frequency; [#]corresponding p-values were calculated by logistic regression analysis; [§]p-value after being adjusted.

Table 5. Association of *IL-8* and breathlessness for tuberculosis.

Polymorphism <i>IL-8</i>	Cases					
	With breathlessness	Without breathlessness	OR (95% CI)	p [#]	AOR (95% CI)	p [§]
Codominant	n=72	n=229				
CC	32 (57.48)	117 (61.88)	1.00 (Reference)	-	1.00 (Reference)	-
CT	37 (39.20)	100 (34.15)	1.35 (0.78-2.39)	0.27	1.39 (0.80-2.42)	0.22
TT	3 (3.32)	12 (3.96)	0.22 (0.06-0.75)	0.01	0.21 (0.06-0.74)	0.01
Dominant	n=72	n=229				
CC	32 (41.8)	117 (49.5)	1.00 (Reference)	-	1.00 (Reference)	-
CT+TT	40 (58.1)	112 (50.4)	0.22 (0.06-0.75)	0.01	0.21 (0.06-0.74)	0.01
Recessive	n=72	n=229				
CC+CT	69 (91.3)	217 (95.01)	1.00 (Reference)	-	1.00 (Reference)	-
TT	3 (3.32)	12 (3.96)	0.78 (0.21-2.86)	0.71	0.80 (0.21-2.97)	0.74

OR, odds ratio; 95% CI, 95% confidence intervals; AOR, adjusted odds ratio; [#]corresponding p-values were calculated by logistic regression analysis; [§]p-value after being adjusted.

patients expressing the mutant allele have 3.66 fold and 1.092 fold of getting hemoptysis and fever, and patients carrying the heterozygous allele have 1.8 fold of having weight loss. Whereas mutant and heterozygous allele shows a borderline risk of night sweats and cough, *i.e.*, 1.1 fold and 1.2 fold. However, patients with the alleles mentioned earlier are protected from chest pain symptoms.

Combinatorial analysis of the risk associated with *IL-4RA* and *IL-8*

The genotypic combinations of the different genotypes were evaluated to see if there was a significant difference in frequency between cases and controls to evaluate SNP-to-SNP interactions. It includes individuals with heterozygous and mutant genotypes combined for the *IL-8* and *IL-4RA* genotypes polymorphism and individuals with wild genotypes for the SNPs. AOR and 95% CI and the genotypic frequency of these combinations are tabulated in *Supplementary Table 1*. We merged the heterozygous and mutant genotypes for both SNPs into a single group. We contrasted it with the wild-type genotype, which served as the standard. (AOR=0.929, 95% CI=0.58-1.47, p=0.75) The patients with the (H+M) genotype did not correlate significantly with TB. Further, the stratification was done based on gender to evaluate the risk of TB. However, no such association was seen for both males (AOR=0.92, 95% CI=0.50-1.71, p=0.80). and female (AOR=0.60, 95% CI=0.29-1.23, p=0.16). Another method used to study the interaction based on risk alleles was evaluating different combinations of risk alleles, as shown in Table 6. None of the alleles exhibits any risk towards TB compared to the zero risk allele used as a reference except three risk allele, which shows a significant protective effect towards TB (AOR=0.38, 95% CI=0.16-0.88, p=0.02) as shown in Table 6.

Discussion

TB is a communicable disease, and despite the significant development in preventing infectious diseases, it remains one of the most important causes of mortality. The widespread infection over time indicates powerful evolutionary pressures in the interaction between host and pathogen genomes. This susceptibility to TB and host genetic factors has been extensively studied using case-control, candidate genes, and genome-wide linkage methods. Many essential candidate genes, like *HLA* genes such as cytokines and their receptors, chemokines and their receptors, pattern recognition receptors (toll-like receptors), mannose-binding lectin, and the dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin), solute carrier family 11A member1 and purinergicP2X7 receptor gene polymorphisms have been associated with differential susceptibility to TB in various ethnic populations. The current study determined the SNPs with *IL-4*, *IL-4RA*, and *IL-8* and their susceptibility to pulmonary TB.

IL-4RAQ576R polymorphism and its relationship with tuberculosis

Previous studies suggest that viral or mycobacterial infection may suppress immunoglobulin E (IgE) levels by inhibiting a Th2 immune response. The fact that *Mtb* infection causes a type-1 immune response has been substantiated by a study. Directly in opposition, intestinal parasites cause a type-2 reaction, and these two reactions -type-1 and type-2- inhibit each other and subsequently lower serum IgE, an indication of a type-2 reaction [12]. A cytokine of the Th2 subtype, IL-4, can promote the Th2 immune response. Numerous studies have shown that the rs1801275 G allele, or the IL-4R allele, is associated with higher levels of IL-4R when arginine is present at position 576 in place of glutamine. This is because rs1801275 (A/G) is a gain of function mutation that accelerates signal transduction. After all, it is located next to the crucially important tyrosine-2 residue, which is a component of the docking site for STAT6, and that is why it can predominate the Th2 immune response pattern [13]. Several diseases, including rheumatoid arthritis [14], atopic problems [15], asthma, and many others have been linked to polymorphic variants of the *IL4RA* gene [16-19].

To our knowledge, however, only a small number of studies have connected the *IL4RAQ576R* polymorphism to TB. Hence, this study evaluated the relationship between *IL-4RAQ576R* and TB patients. When all three models were applied, it was observed that in the codominant model, when the mutant allele was compared with the wild type, a significant association was seen, and the mutant allele, *i.e.*, *IL-4RA 576R* showed a significant protective role towards TB. The notion above is supported by the fact that *IL-4RA 576R* demonstrates a considerable protective role in the recessive model. Also, previous studies have hypothesized that TB is not due to a lack of Th1 but due to a decreased Th2-like response, including IL-4, and our results have also supported this fact as rs1801275 modulates the binding of IL-4 with its receptor which is a major contributor to Th2 like a response [20].

Relation of *IL-4RAQ576R* polymorphism in association with clinical symptoms

We also investigated the role of the *IL-4RAQ576R* polymorphism in clinical symptoms such as cough, breathlessness, appetite loss, weight loss, chest pain, hemoptysis, fever, and night sweats. To the best of our knowledge, this is the first study illustrating the contribution of *IL4RAQ576R* polymorphisms to symptoms in TB. The codominant model showed a substantial correlation between cough and the heterozygous allele compared to the wild type. The link was substantial when the dominant model was used, with a 2.40-fold increased likelihood of cough in TB patients. Similarly, no significant results were found when codominant and dominant models were applied to breathlessness, lack of appetite, weight loss, and chest pain, but all of these symptoms had marginally protective effects in TB patients. However, when the same models were applied

Table 6. Combined effects of *IL-4RA* (A/G) and *IL-8* (C/T) on the risk of tuberculosis.

No. of risk allele	Controls	Cases	OR	p [#]	AOR (95% CI)	p [§]
0	82	86	1.00 (Reference)	-	1.00 (Reference)	-
1	121	139	1.09	0.6459	1.21 (0.77-1.89)	0.71
2	76	67	0.84	0.4459	0.85 (0.54-1.33)	0.48
3	22	9	0.39	0.0266	0.38 (0.16-0.88)	0.02

OR, odds ratio; AOR, adjusted odds ratio; [#]corresponding p-values were calculated by logistic regression analysis; 95% CI, 95% confidence intervals; [§]p-value after being adjusted; 0, (C/C, A/A); 1, (C/C, A/G) (C/T, A/A); 2, (C/T, A/G) (T/T, A/A), (C/C, G/G); 3, (C/T, GG) (T/T, A/G).

to hemoptysis and fever, a somewhat increased risk for these symptoms was seen, but no significant results were discovered.

IL-8 +781 C/T polymorphism and its relationship with tuberculosis

Leukocytes are attracted to inflammatory areas by IL-8. Leukocytes produce and release it in response to Mtb or its constituent parts. The IL-8 found on chromosome 4q13-21 consists of four exons, three introns, and the proximal promoter. Several polymorphisms have been discovered in the *IL-8* gene. An increased cytokine IL-8 has been linked to a polymorphism (rs2227306) in the promoter region (Intron 1) of the gene for IL-8, which is 781 C/T. The T allele is coupled to a preference for C/EBP β binding in lung epithelial cells. C/EBP β binds the *IL-8* gene's regulatory region, a crucial inflammation regulator that significantly boosts transcription [20-22]. Many studies have linked various SNPs to TB, even in IL-8 [23,24], but very few have investigated rs2227306 with TB. Our study investigated the relationship between *IL-8* +781 C/T polymorphism and TB. Our research revealed a significant association between the mutant allele T and the wild-type C allele, indicating that the T allele of IL-8 at the +781 location has a protective effect on TB (OR=0.49, p=0.03, 95% CI=0.24-0.96). Many studies have demonstrated that the C allele increases the production of IL-8 and the susceptibility to the disease. The highly produced IL-8 may encourage polymorphonuclear leukocyte apoptosis, and then induce inflammation. Additionally, the increased expression of IL-8 draws an excessive number of leukocytes to the lesion site, where they produce proteases, elastases, and free radicals that significantly damage the tissue.

Relation of IL-8 +781 C/T polymorphism in association with clinical symptoms

We have also correlated all the symptoms discussed earlier to *IL-8* +781 C/T. All three models were applied, and instead of breathlessness, none of the symptoms exhibited a significant correlation with *IL-8* +781 C/T in TB patients. The T allele shows a protective effect towards symptoms like breathlessness, loss of appetite, and chest pain in TB patients. But the heterozygous allele, compared to the wild type in codominant, shows the risk of appetite loss and breathlessness in TB patients. In comparison, the T allele has been associated with an increased risk of cough in TB patients in codominant (1.80-fold), dominant (1.26-fold), and recessive (1.64) models. When the codominant model was applied to evaluate the co-relation of risk of loss of weight with *IL-8* +781 C/T, it was found that the heterozygous genotype shows a borderline protective effect.

In contrast, the T allele increases the risk of weight loss by 1.73-fold and 1.86-fold in the recessive model. Also, the T allele has been shown to increase hemoptysis by 3.66-fold using the codominant model and 2.77-fold in the recessive model, whereas wild type and heterozygous decrease these risks compared to mutant type. Fever and night sweats also enhanced, as the T allele increased these symptoms by 1.92 and 1.11-fold when the codominant model was applied.

Combinatorial analysis of the risk associated with rs1801275 and rs2227306

We have employed two methods in our study to evaluate the collective risk of these SNP towards TB by employing different combinations possible for both. Many studies have investigated the asso-

ciation between various SNPs [25], but till now, no such association has been evaluated in TB for these. When heterozygous and mutant genotypes common in both the SNPs were compared with the wild type, no significant associations were found, even when applied separately to males and females. But all of them show a protective effect; even in females, the odds were slightly lower than in males. In another method, various combinations of the risk allele were made and compared to the zero risk allele, *i.e.*, wild type of both the SNPs, but except for the three-risk allele, which shows a significant association, none shows a significant association. However, it is interesting to note that as the number of risk alleles increases, the protective effect increases, and the 3-risk allele shows a significant outcome.

Limitations of the study

It is essential to mention that the present study has certain limitations. One of the key impediments to our research is the small sample size. Our findings regarding the association of *IL4RAQ576R* and *IL-8* +781 C/T risk were restricted to the North Indian population. Due to ethnic or regional differences, the conclusions may vary in different countries or regions. A thorough analysis of extended haplotypes across candidate genes must also be combined with functional studies of gene and protein function to determine which polymorphisms are disease-associated. Therefore, to confirm the findings, large, extensive case-control studies involving people of different nationalities and ethnicities are precious for understanding how TB develops and may aid in early disease prevention.

Strengths of the study

On the other hand, the present study has certain advantages. To the best of our knowledge, this is the first study illustrating the contribution of *IL4RAQ576R*, *IL-8* C/T TB polymorphisms to TB in the North Indian population. Our study is the first to assess and report the stratifications between the clinical indicators pertaining to TB and *IL4RAQ576R*, *IL-8* C/T polymorphism. Additionally, we have used explicit criteria for study inclusion and a strict procedure for data extraction.

Conclusions

In our study, *IL-4 RA 576R* and *IL-8* +781 C/T showed a significant protective role towards TB. Whereas when symptoms were evaluated, TB patients showed a significant association, only towards cough in *IL-4RA576R* and breathlessness in *IL-8* +781 C/T. Further on combined effects of both the SNPs were evaluated and 3-risk allele shows a significant association with TB.

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Online supplementary material:

Supplementary Table 1. Combinatorial analysis of IL-4RA and IL-8.