



ASSOCIATION OF CYTOKINE POLYMORPHISMS AND GENE COMBINATIONS WITH SUSCEPTIBILITY TO PULMONARY TUBERCULOSIS

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Abstract: Background: Mycobacterium tuberculosis is a known to cause chronic tuberculosis with high morbidity and mortality, especially in developing countries. Genetic variability of the host determines the susceptibility to the tuberculosis infection. The present study was to evaluate the association of genetic polymorphism among cytokines, also to evaluate the effect of different gene combinations of IFN γ gamma and its regulating cytokine genes. **Aim:** To evaluate the presence of single nucleotide polymorphism associated with the genes IFN γ (+874 A/T), TNF α (-308 G/A), IL-10 (-1082 G/A) among the tuberculosis patients compared with the healthy human controls, as well as study of IFN γ gene combination with IL-10 and TNF α in Hyderabad region of the Southern part of India. **Materials and Methods:** A case control study was conducted, genomic DNA was extracted from peripheral blood samples from both TB confirmed cases and from healthy controls. The association of single nucleotide polymorphism in IFN γ (+874 A/T), TNF α (-308 G/A), IL-10 (-1082 G/A) was investigated by polymerase chain reaction amplification refractory mutation system. (ARMS-PCR). IFN γ gene (+874 A/T) functional single nucleotide polymorphism combinations in TNF α (-308A/G), IL-10 (-1082 A/G) were analyzed. A total of 155 healthy controls and 150 cases were included in the study. **Results:** We found TNF α (-308A/G), GG genotype (OR-0.423, 95% CI-0.262-0.682, p=0.001) was significantly associated with the tuberculosis incidence. No significant correlation between IFN γ (+874 A/T) A or AA, IL-10 (-1082 A/G) G or GG, allele or genotype respectively in tuberculosis patients was seen. A multi gene combination study, we found combination of IFN γ TA^{ln} IL-10 AA^{hi} (OR-1.63, 95% CI- 0.01-2.64, p=0.043) and IFN γ TA^{ln}- TNF α GG^{low} (OR-4.14, 95% CI-2.31-7.42, p=0.00) were associated with the tuberculosis cases. **Conclusion:** From our study we found that genetic variability TNF α (-308A/G), GG genotype and multi gene combination IFN γ TA^{ln} IL-10 AA^{hi} and IFN γ TA^{ln}- TNF α GG^{low} are associated with tuberculosis infection.

Keywords: Pulmonary Tuberculosis, Cytokines, Single nucleotide polymorphism

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INTRODUCTION

A decades long fight to contain the tuberculosis infection through combine efforts of government and private agencies has lead to the decline in tuberculosis especially in developed and industrialized nations. Unfortunately, developing countries still waging a war to prevent the spread of this chronic infection especially in a large populated countries like India. India is one of the top countries having a large number of people suffering with

tuberculosis. According to Global TB report 2021, the estimated incidence cases of TB in India were around 188 per lakh population, ¹ accounts of a 26% of of global burden of tuberculosis.²

Several factors such as individual immunity, its response to the infection, cytokines level and genetic factors play an important role in restricting the disease progress. Cytokines play a pivotal role, release of several pro-inflammatory cytokines derived from T cells, prevent the multiplication of the MTB in the macrophages. The major factors which effect release of cytokines depends upon the genetic makeup of the individual and cytokines genes associated with single nucleotide polymorphism (SNP) at the coding region determines the release of particular cytokine.³

A pro-inflammatory cytokine Interferon gamma (IFN γ), play an important role against MTB infection, through activation of macrophages leading to microbicidal response against MTB. IFN γ has been known to secreted by several immune cells including T-cells, natural killer cells and macrophages. IFN γ known to induce the production of nitric oxide and ROI's leading to restriction and killing of MTB. ⁴ IFN γ (+874 A/T) SNP known to have a profound effect on the production of this cytokine and has variable effect in different population.⁵

TNF α is a proinflammatory cytokines, secreted by different immune cells such as T cells, macrophages, neutrophils and mast cells. It mainly plays a role in regulation of

inflammatory response, IFN γ production. ⁶ Synergic action of TNF α along with IFN γ known to initiate the production of reactive nitrogen intermediates leading to the activation of the bacteriostatic action of macrophages as well as stimulating the migration of the immune cells to the site of infection leading to the granuloma formation. ⁷ TNF α (-308 G/A) has been associated with the severity of the tuberculosis.⁸

IL-10 is one of immunoregulatory cytokines, which is known to down regulate the proinflammatory cytokines. The main action of this cytokine is to prevent the collateral damage and collapse of the lung, but it has inverse action against the IFN γ and TNF α leading to the activation and multiplication of the TB bacilli.⁹ The IL-10 (-1082 G/A) polymorphism in Ethiopia and found that it has profound effect on the level of IL-10 circulating in the blood, and known as one of the predisposing factors for the development of tuberculosis.⁸

In the present study we aim to evaluate the presence of SNP associated with the genes IFN γ (+874 A/T), TNF α (-308 G/A), IL-10 (-1082 G/A) among the tuberculosis patients compared with the healthy human controls at Hyderabad region of the Southern part of India. The complexity of immune reactions and interactions among the different cytokines affected by the polymorphisms, the study of multi-gene combination gives a better understanding. In the present study we also aim to identify selected combinations of IFN γ cytokine gene with other cytokine genes including TNF α and IL-10 to determine the occurrence of tuberculosis.

MATERIALS AND METHODS

Study design: Case-control study

Study group: For the study group the Inclusion criteria was included all the TB cases with presence of clinical signs and symptoms of tuberculosis along with typical chest radiological findings, along with positive sputum culture, positive for acid-fast staining, the exclusion criteria was the subjects with diabetes, cancer, cardiac abnormalities and other infectious diseases. For healthy controls the Inclusion criteria were the subjects without any comorbidities like diabetes, immunocompromised state and other infectious diseases and exclusion criteria was the patients with comorbidities including chronic infections, diabetes mellitus, inflammatory and autoimmune diseases. The study

Table 1. shows the gene, primer sequence of ARMS-PCR

| Gene | Primer Sequences 5'-3' (ARMS PCR) | Annealing temperature | Amplicon size | Reference |
|-------------------------------|---|-----------------------|---------------|---------------|
| IFN γ (+874 A/T) | Allele A -5'-TTC TTA CAA CAC AAA ATC AAA TCA-3' Allele T - 5'-TTC TTA CAA CAC AAA ATC AAA TCT-3' COMMON PRIMER-5'-TCA ACA AAG CTG ATA CTC CA-3, | 62 | 261 bp | ¹⁰ |
| TNF α (-308G/A) | Allele G 5'-ATA GGT TTT GAG GGG CAT GG -3' Allele A 5'- ATA GGT TTT GAG GGG CAT GA-3' COMMON PRIMER 5'-TCT CGG TTT CTT CTC CAT CG-3' | 60 | 186 bp | ¹¹ |
| IL-10 (-1082 G/A) | Allele G 5'-TACTAAGGCTTCTTTGGGAG-3' Allele A 5'- CTACTAAGGCTTCTTTGGGAA-3' COMMON PRIMER 5'- CAGCCCTTCCATTTTACTTTC-3' | 62 | 550 bp | ¹² |

was approved by the Institutional Ethics Committee of Kamineni Academy of Medical Sciences and Research Centre (KAMSRC/IEC/24/2018) and was conducted at Department of Microbiology for a period of three years from 2108-2021. A written consent was taken from all participants along with demographic data, the questionnaire collected information regarding smoking, frequency of smoking, alcohol consumption and the BCG vaccination.

Statistical analysis: All data were presented as the mean standard deviation (SD) for quantitative variables or percentages for categorical variables. The genotypic and allelic frequencies were compared using a chi-square test or Fisher's exact test between case and control groups. P value of < 0.05 were considered significant for both Pearson and Fisher's exact tests. The odds ratio (OR) and 95% confidence interval (CI) for allele and genotype forms were calculated by univariate and multinomial logistic regression was applied respectively. Hardy-Weinberg Equilibrium (HWE) was determined by applying the equation (p²+2pq+q²). IFN- γ +874 T/A genotype combinations with IL-10 -1082 A/G, TNF- α -308G/A and were used to determine two gene combination effect. Statistical analysis was performed using SPSS software.

In the present study nine possible genotype combinations were derived with IFN γ genotypes for each of the cytokine genotypes. These combinations were then compared between controls and tuberculosis patients using Pearson χ^2 or Fischer's exact test

DNA extraction and PCR: Peripheral blood sample 3-5ml were collected in vacutainers EDTA tubes from patients and normal individuals. DNA extraction was done by whole blood DNA extraction kit (Nucleospin, Microbial DNA, Germany) according to the manufacturer's instructions, and stored at 20 °C.

Polymerase chain reaction (PCR): The PCR (Takara gradient thermal cycler dice. Japan) has been carried out in a form of ARMS-PCR (Amplification refractory mutation system-Polymerase chain reaction). The individual primer sequence of all the genes under study along with annealing temperature and amplified product base pair size has been mentioned in table 1.

RESULTS

Study Population: A total of 150 confirmed TB cases and 155 healthy controls were included in the present study. The mean age of the TB patients a mean of 47.03(min-18 max-

85 years) and mean age of the controls is 39.4(min-19 max-78 years) The details of the age, gender has been mentioned in table 2.

Table 2. Patients details of demographic data along with confounding factors of TB patient and control group

| Variables | | TB Paitents | Control group |
|----------------|----------------|-------------|---------------|
| Gender | Female | 99 | 51 |
| | Male | 74 | 81 |
| BCG Vaccine | Vaccinated | 112 | 141 |
| | Non-Vaccinated | 38 | 14 |
| smoking | Smokers | 16 | 4 |
| | Non- smokers | 134 | 151 |
| Alcohol | Yes | 22 | 3 |
| | No | 128 | 152 |
| Married status | Yes | 136 | 137 |
| | No | 14 | 18 |

Table 3: Frequencies of alleles, genotypes and odds ratios regarding different SNP'S

| Types of SNP | | | Cases | Controls | P value | Odds ratio | 95% CI | |
|-----------------------|-----------|----|-------|----------|---------|------------|--------------------|------------------|
| | | | | | | | Lower Limit | Upper limit |
| IFN γ +874 T/A | Allele | A | 135 | 110 | 0.128 | 1.086 | 0.757 | 1.557 |
| | | T | 248 | 231 | | | | Reference Allele |
| | Geno type | TA | 98 | 76 | 0.108 | 0.638 | 0.376 | 1.081 |
| | | TT | 15 | 34 | 0.14 | 1.864 | 0.883 | 3.934 |
| AA | | 37 | 45 | | | | Reference Genotype | |
| TNF α -308 G/A | Allele | A | 71 | 109 | 0.01* | 0.77 | 0.622 | 0.953 |
| | | G | 148 | 141 | | | | Reference Allele |
| | Geno type | AA | 2 | 14 | 0.0001* | 0.083 | 0.018 | 0.382 |
| | | AG | 69 | 95 | 0.001* | 0.423 | 0.262 | 0.682 |
| GG | | 79 | 46 | | | | Reference Genotype | |
| IL10 -1082 G/A | Allele | A | 142 | 151 | 0.92 | 1.001 | 0.807 | 1.242 |
| | | G | 61 | 65 | | | | Reference Allele |
| | Geno type | AA | 89 | 90 | 0.373 | 0.494 | 0.144 | 1.801 |
| | | AG | 53 | 61 | 0.231 | 0.434 | 0.124 | 1.524 |
| GG | | 8 | 4 | | | | Reference Genotype | |

Genotyping of IFN γ (+874 T/A)

The allele frequency between A and T between TB infection cases and controls was not statistically significant (p=0.128) (Table 3). Genotype heterozygous TA was most frequent genotype found in TB cases and in healthy controls, though the difference between the cases and

controls were not statistically significant, followed by AA and TT, This results indicate that TA genotype has been associated with the TB infection (OR-0.638, 95%CI-0.376-1.081, p=0.108), than the TT genotype (OR-1.864, 95% CI-0.883-3.934, p=0.14), (Figure 1, Table 3)

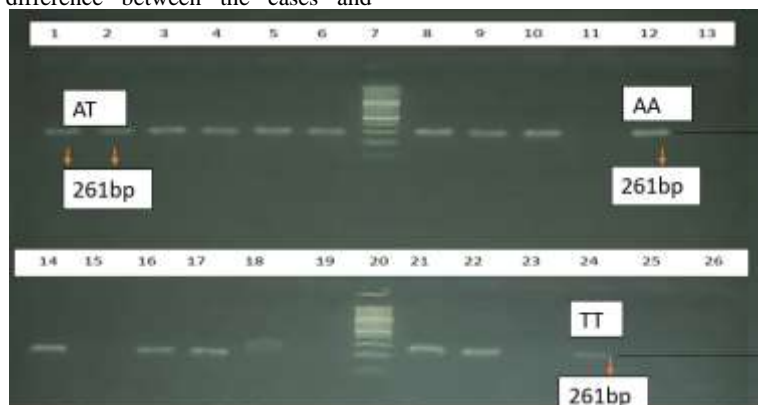


Figure 1: ASO-PCR for the detection of IFN-Gamma (+874T/ A).

Lanes: AA- homozygous- lane 10-11,12-13,14-15.
 Allele AT- Heterozygous – Lane- 1-2,3-4,5-6,8-9,16-17,21-22.
 Allele TT homozygous- Lane 23-24;
 Lane 7 and 20 100 bp ladder

frequency of TNF α (-308A/G), G (mutant type) is found more in TB patients compared to the controls (OR-0.77, 95% CI- 0.622-0.953, p=0.01) (table 3). In addition, GG genotype frequency was more in patients compared to controls (OR-0.423, 95% CI-0.262-0.682, p=0.001). This result indicates alleles G and genotype GG has been significantly associated with the TB than allele A and genotype AG.

Genotyping of TNF α (-308A/G)

TNF α is known to induce granuloma formation, the allele

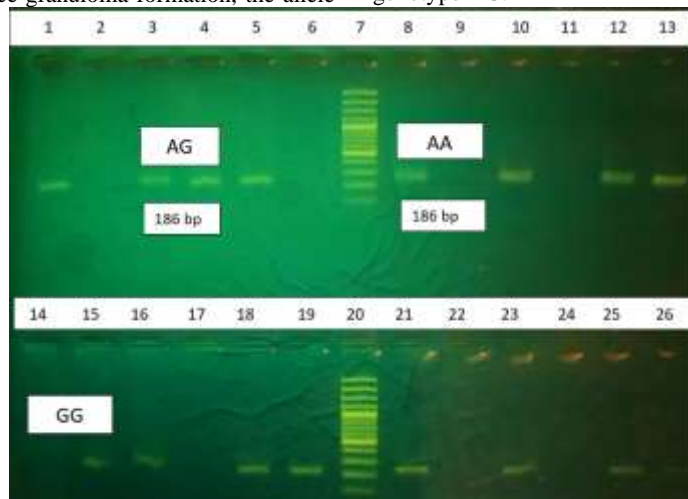


Figure 2: ASO-PCR for the detection of TNF α -308A/G.

Lanes: AA- homozygous- lane 1-2,5-6,8-9,10-11,16-17,21-22,23-24.
 Allele AG- Heterozygous – Lane- 3-4,12-13,18-19,25-26.
 Allele TT homozygous- Lane 14-15.
 Lane 7 and 20 100 bp ladder

balance adequately between inflammatory and immunopathological response. In the present study we found no significant genotype associated with disease. Frequent genotype was AA found in both groups (OR-0.494, 95% CI-0.144-1.801, p=0.373), followed by AG (OR-434, 95% CI-0.124-1.524, p=0.231). Allele frequency A found more in controls, though not significant (OR-1.001, 95% CI-0.807-1.242, p=0.92).

Genotyping of IL-10 (-1082 A/G)

IL-10 is known to acts an inhibitory cytokine and acts to

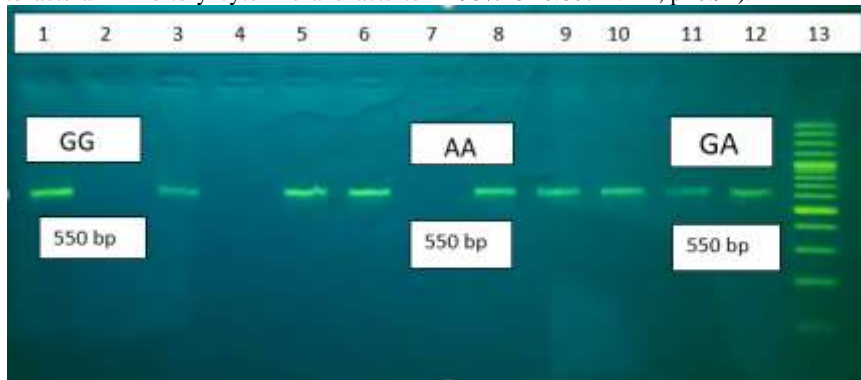


Figure 3: ASO-PCR for the detection of TNF α -308A/G.

Lanes: AA- homozygous- lane 4-5,7-8.
 Allele GA- Heterozygous – Lane- 5-6,9-10,11-12.
 Allele GG homozygous- Lane 1-2,3-4;
 Lane 13- 50 bp ladder

We found no significant association between the IL-10 -1082 A/G and the pulmonary tuberculosis, but the combination of IFN γ TA^{hi} IL-10 AA^{hi} resulted increased risk of tuberculosis (OR-1.63, 95% CI- 0.01-2.64, p=0.043). The statistical significance has been achieved after Bonferroni corrections applied. (Table 4).

Combinations of IFN γ (+874 T/A) T/A with IL-10 -1082 A/G SNPs

Table 4. Genotypes combination analysis of IFN γ and IL-10

| IFN γ TO IL10 | CASES | CONTROLS | Chi Square | Odds ratio | | | P value |
|----------------------|------------|-------------|------------|------------|-------|-------|---------|
| | | | | estimated | lower | upper | |
| TT-AA | 7 25.9% | 20 74.1% | 6.40 | 0.33 | 0.13 | 0.80 | 0.013 |

| | | | | | | | |
|-------|-------|--------|------|------|------|-------|-------|
| TT-AG | 8 | 13 | 1.10 | 0.61 | 0.24 | 1.53 | 0.292 |
| | 38.1% | 61.9% | | | | | |
| TA-GG | 0 | 1 | 0.96 | 0 | -1 | -1 | 0.326 |
| | 0.0% | 100.0% | | | | | |
| TA-AA | 59 | 44 | 4.08 | 1.63 | 1.01 | 2.64 | 0.043 |
| | 57.3% | 42.7% | | | | | |
| TA-GA | 35 | 30 | 0.71 | 1.26 | 0.73 | 2.19 | 0.396 |
| | 53.8% | 46.2% | | | | | |
| TA-GG | 4 | 2 | 0.74 | 2.09 | 0.37 | 11.61 | 0.386 |
| | 66.7% | 33.3% | | | | | |
| AA-AA | 23 | 26 | 0.11 | 0.89 | 0.48 | 1.65 | 0.731 |
| | 46.9% | 53.1% | | | | | |
| AA-GA | 10 | 18 | 2.23 | 0.54 | 0.24 | 1.21 | 0.134 |
| | 35.7% | 64.3% | | | | | |
| AA-GG | 4 | 1 | 1.93 | 4.21 | 0.46 | 38.19 | 0.164 |
| | 80.0% | 20.0% | | | | | |
| Total | 150 | 155 | | | | | |
| | 49.2% | 50.8% | | | | | |

Combinations of IFN γ (+874 T/A) T/A with TNF α -308 G/A SNPs

Along with the IFN γ , TNF α play an important role in the granuloma formation, disease localization and prevention of the spread of the infection. The gene combination studies

for these two cytokines, when evaluated we noted that IFN γ TA^{ln}- TNF α GG^{low} (OR-4.14, 95% CI-2.31-7.42, p=0.00) in tuberculosis cases, has been associated with the tuberculosis (Table 5)

Table 5: Genotypes combination analysis of IFN γ and TNF- α

| IFN γ TO TNF α | CASES | CONTROLS | Chi Square | Odds ratio | | | P value |
|------------------------------|-------|----------|------------|------------|-------|-------|---------|
| | | | | estimated | Lower | upper | |
| TT-AA | 0 | 3 | 2.93 | 0 | -1 | -1 | 0.086 |
| | 0.0% | 100.0% | | | | | |
| TT-GA | 10 | 20 | 3.34 | 0.48 | 0.21 | 1.06 | 0.067 |
| | 33.3% | 66.7% | | | | | |
| TT-GG | 5 | 11 | 2.17 | 0.45 | 0.15 | 1.33 | 0.140 |
| | 31.3% | 68.8% | | | | | |
| TA-AA | 2 | 6 | 1.92 | 0.33 | 0.06 | 1.68 | 0.165 |
| | 25.0% | 75.0% | | | | | |
| TA-GA | 41 | 51 | 1.12 | 0.76 | 0.46 | 1.25 | 0.289 |
| | 44.6% | 55.4% | | | | | |
| TA-GG | 55 | 19 | 24.71 | 4.14 | 2.31 | 7.42 | 0.00 |
| | 74.3% | 25.7% | | | | | |
| AA-AA | 0 | 5 | 4.91 | 0 | -1 | -1 | 0.026 |
| | 0.0% | 100.0% | | | | | |
| AA-GA | 18 | 24 | 0.77 | 0.85 | 0.59 | 1.23 | 0.377 |
| | 42.9% | 57.1% | | | | | |
| AA-GG | 19 | 16 | 0.41 | 1.26 | 0.62 | 2.55 | 0.520 |
| | 54.3% | 45.7% | | | | | |
| Total | 150 | 155 | | | | | |
| | 49.2% | 50.8% | | | | | |

DISCUSSION

Understanding the genetic makeup of the individual and gene polymorphisms of different genes will be added milestone in the era of the personalized treatment. Proinflammatory cytokines play a important role in protection against the tuberculosis and the anti-inflammatory cytokines maintain a balance between the inflammation and prevents the tissue damage. Host genetic factors including the different cytokine gene polymorphism play an important role in determining the individual susceptibility or resistance to pulmonary tuberculosis.¹³

IFN γ SNP (+874 T/A) has been located at the 5' end of CA repeat of first intron. Point mutations leads to the low production of this cytokines.¹⁴ In the present study we found atwe found high TA genotype frequency in patients compared to controls, analysis found TA causes increase in susceptibility to the tuberculosis. Our findings are in the concordance with Lucia et al¹⁵ and studies from Mansouri et al¹⁰ and a study conducted on Iranian population.¹⁶ High presence of genotype TA, indicates intermediate production of interferon gamma leading less activation of macrophages, cell proliferation., leading to favourable conditions for the tubercle bacilli intracellular multiplication and disease progress. Our results are contrary to the studies found in

China,¹⁷ and Adane et al⁸ where they found high existence of A allele and AA genotype compared to our study, we found high existence of T allele with TA genotype. This disparity of different genotypes and different alleles in different global studies might be due to the different ethnicity and different origin, along with disparity in sample size collections.

In controlling the mycobacterial infection, TNF α play a primordial role activating important cells including macrophages, T lymphocytes and contributes granuloma formation.¹⁸ In the present study, G allele and GG genotype frequency was higher in patients compared to controls, leading low production, inhibition of migration of different immune cells which acts against the TB bacilli. The presence of GG genotype promotes lack of formation of functioning granuloma, as well as promoting the dissemination of the bacilli.¹⁹ Our results were in found similar with the study conducted by Kurdistani et al, found similar high allele frequency of G and genotype GG in Iranian population.²⁰ A study from Fan al²¹ and Vijaykumar et al²² has found not any significant difference of G alleles and GG genotypes among controls and patients contrary to our results, this might be due to different ethnic population and sample size.

IL-10 primary considered as an inhibitory cytokine, it maintains inflammatory response as well as indirectly favours the survival of TB bacilli as well leading to increase in the incidence of tuberculosis.²³ In the present study of evaluation of IL-10 -1082 G/A, we found high frequency of A allele with AA genotype both in patients and controls, followed by AG. Our results were similar with the study conducted in Korea, where no significant difference has been noticed between TB patient group and health controls.²⁴ Contrary to our results G a study conducted in Turkey found that G allele frequency has been associated with tuberculosis patients.²⁵ According to Adane et al it was stated that presence of A allele and AA genotype at -1082 position has a minor effect in downregulating Th1 proinflammatory cytokines and less interruption of host immune response.⁸ Echoing similar statement our results found high A allele and AA genotype, indirectly not effecting much on the immune status both in patients and in healthy controls.

We evaluated different genotype combination analysis out of which only 2/18 possible combinations showed a significant association, with the increased risk of tuberculosis. We found that IFN γ TA^{ln} IL-10 AA^{hi} has been associated with tuberculosis, contrary to the study conducted by Ambreen Ansari et al where they found IFN γ ^{hi} IL10^{lo} showed association with pulmonary advanced disease this implies that different genotype combinations associated with the disease depends upon the ethnicity and sample size collection.²⁶ A study from Pakistan²⁶ found that gene combination IFN γ with TNF α showed only weak association, contrary to their study our study found that IFN γ TA^{ln}- TNF α GG^{low} combination was significantly associated with the tuberculosis cases, which implies that different combinations of different genotypes are either associated or not related with the tuberculosis.disease.

CONCLUSION

Tuberculosis is a multifactorial disease, involving immunological and environmental and confounding factors. Single nucleotide polymorphism in the cytokines helps in identifying the persons at risk of developing tuberculosis.

Our results demonstrated that polymorphisms in IFN γ , TNF- α has been associated with risk of developing disease and further progressing of pulmonary tuberculosis. We haven't found any correlation of IL-10 polymorphism with the disease. Our association studies highlighted the importance of case stratification of the patient group. Further studies are warranted to study SNP in larger group of population with higher number of sample size as well as additional multi-loci gene interaction studies in different ethnic groups to evaluate and identify the persons at risk of developing chronic tuberculosis infection.

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