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 susceptbility 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 IFNγ gamma and its regulating cytokine genes. **Aim:** To evaluate the presence of single nucleotide polymorphism associated with the genes IFNγ (+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 IFNγ (+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 polymorphismcombinations in TNFα (-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 TNFα (-308A/G), GG genotype (OR-0.423, 95% CI-0.262-0.682, p=0.001) was significantly associated with the tuberculosis incidence. No signficant correlation between IFNγ (+874 A/T) A or AA , IL-10 (-1082 A/G) G or GG , allele or genotype respecitvley in tuberculosis patients was seen. A multi gene combination study, we found combination of IFN γ TA ^{In} IL-10 AA ^{hi} (OR-1.63, 95% CI- 0.01-2.64, p=0.043) and IFNγ TA ^{In}- TNFα 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 TNFα (-308A/G), GG genotype and multi gene combination IFN γ TA ^{In} IL-10 AA ^{hi} and IFNγ TA ^{In}- TNFα 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 nucelotide polymorphism (SNP) at the coding region determines the release of particular cytokine. [3]

A pro-inflammatory cytokine Interferon gamma (IFNγ), play an important role against MTB infection, through activation of macrophages leading to microbicidal response against MTB. IFNγ has been known to secreted by several immune cells including T-cells, natural killer cells and macrophages. IFNγ known to induce the production of nitric oxide and ROI's leading to resitriction and killing of MTB. [4] IFNγ (+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, secrted by different immune cells such as T cells, macrophages, neutrophils and mast cells. It mainly plays a role in regulation of

inflammatory response, IFNγ production. [6] Synergic action of TNF α along with IFNγ 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 IFNγ and TNF α leading to the activation and multiplication of the TB bacilli.[9]. The IL-10 (-1082 G/A) polymorphism in Ethopia 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 IFNγ (+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 IFNγ 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 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 combinatins were derived with IFNγ genotypes for each of the cytokine genotypes. These combinationswere then compared between controls and tuberculosis patients using perason x^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.

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

Gene	Primer Sequences 5'-3' (ARMS PCR)	Annealing temperature	Amplicon size	Reference
IFNγ (+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]
TNFα (-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]

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

							95% CI	
Types of SNP			Cases	Controls	P value	Odds ratio	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
TNFα-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). Gentoype 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)

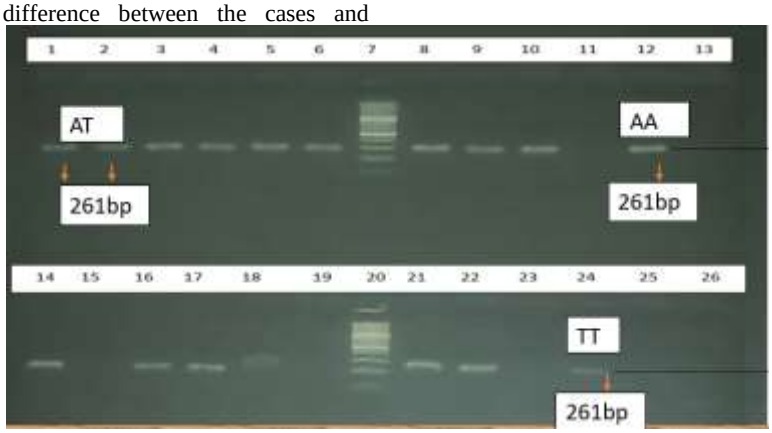

Figure 1: ASO-PCR for the detection of IFN-Gamma (+874T/ A).

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

Genotyping of TNFα (-308A/G)

TNF α is known to induce granuloma formation, the allele 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.

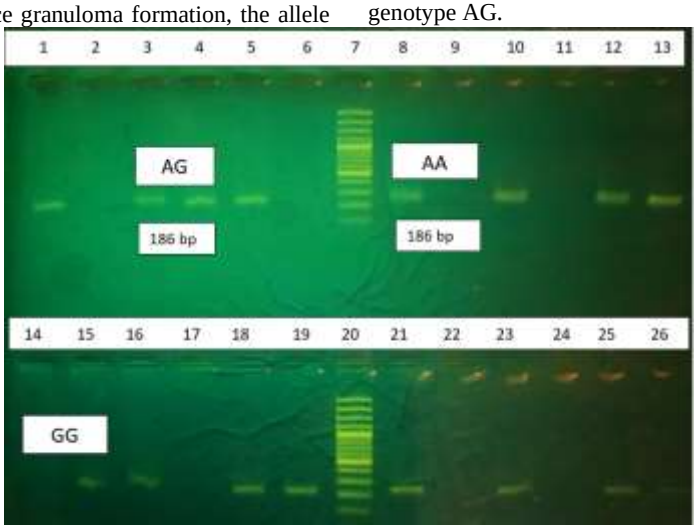

Figure 2: ASO-PCR for the detection of TNFα-308A/G.

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

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

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

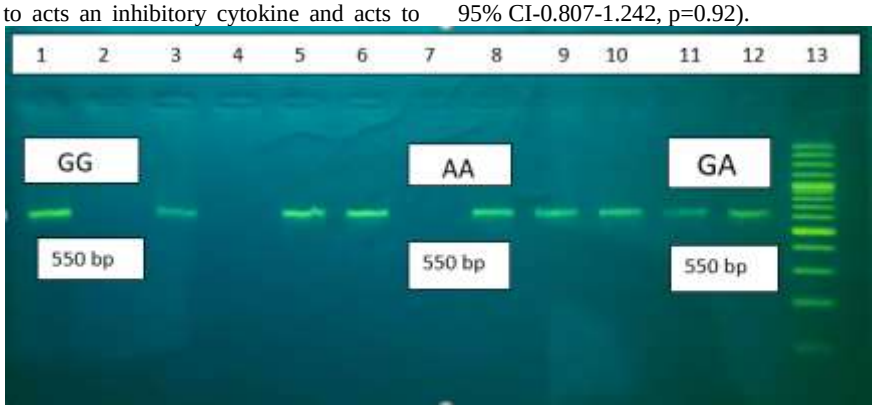

Figure 3: ASO-PCR for the detection of TNFα-308A/G.

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

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

We found no significant association between the IL-10 -1082 A/G and the pulmonary tuberculosis, but the combination of IFN γ TA ^{ln} 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).

Table 4. Genotypes combination analysis of IFNγ and IL-10

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

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 IFNγ (+874 T/A) T/A with TNF α -308 G/A SNPs

Along with the IFNγ, TNFα 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 IFNγ TA ^{In}- TNFα 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 IFNγ and TNF-α

IFN ϒ 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. Profinflammatory cytokines play a important role in protection aganist 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]

INFγ 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 al [15] and studies from Mansouri et al [10] 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 al [8] 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, TNFα 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 al [21] and Vijaykumar et al [22] 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 ethinic 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 ^{In} IL-10 AA ^{hi} has been associated with tubreculosis, contrary to the study conducted by Ambreen Ansari et al where they found IFN γ ^{hi} IL10 ^{lo} showed asociation with pulmonary advanced disease this implies that different genotype combinations associated with the disease depends upon the ethinicity and sample size collection. [26] A study from Pakistan [26] found that gene combination IFN γ with TNFα showed only weak association, contrary to their study our study found that IFNγ TA ^{In}- TNFα GG ^{low} combination was significantly associated with the tuberculosis cases, which implies that different combinations of different gentypes 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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