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Association of Interferon-γ+2019A/G Gene Polymorphism Susceptibility to Tuberculosis

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ABSTRACT:

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M.tb*), and primarily affects the lungs. Approximately one-third of the world population is latently infected with tuberculosis, but only 10% of infected individuals develop an active disease. The household contacts (HHC) of active pulmonary Tuberculosis (APTB) patients are more likely to develop TB. Host immunity and genetic factors play an important role in TB infection. Interferon-gamma (IFN-γ) is essential in the immune response against TB, and many studies have explored the relationship between its genetic variations and TB susceptibility across various populations worldwide. In the present study, we examined the single nucleotide polymorphism (SNP) of IFN-γ+2019A/G in TB susceptibility. Genomic DNA was isolated from APTB patients, their HHC and HC. Genotyping for IFN-γ +2019A/G, by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). Our results for IFN-γ+2019A/G Polymorphism, heterozygous AG and homozygous mutant GG genotypes of APTB patients 5 and 3.76 fold risk and heterozygous AG genotype of HHC indicated 4.8-fold risk when compared with healthy controls. G allele is showing a significant risk in both patients and HHCs when compared with HCs. To our knowledge, this is the first report from the South Indian population which is focused on the role of IFN-γ+2019A/G gene polymorphism in the immunopathogenesis of TB. For a better understanding, further studies are required to confirm in a larger sample size and also in various ethnicity groups.

Keywords: Tuberculosis, Household contacts, Interferon-γ, Pro-inflammatory cytokine, SNP, Susceptibility.

INTRODUCTION:

Tuberculosis (TB) is an infectious disease which is caused by an etiologic agent *Mycobacterium tuberculosis (M.tb)*. The global TB epidemic is a leading cause of death nearly 1.3 million individuals and infects more than 10.6 million incidence rates worldwide (WHO2024). About one-third of the world's population has been infected without any symptoms despite the presence of lung lesions, providing a massive reservoir of the pathogen that can reactivate to active pulmonary tuberculosis (APTB) and cause further transmission. (Gordhan et al., 2021). Most symptomatic TB cases develop from long-term asymptomatic infection known as latent tuberculosis infection (LTBI) (Ishikawa et al., 2022). Five to fifteen percent of LTBI patients are prone to developing TB and transmitting the disease during their lifetime, often co-occur with host immune-compromising conditions (Kilinç et al., 2021). LTBI is more prevalent among household contacts (HHC's) than normal individuals (Simmons et al., 2018). Household contacts of TB patients had an average 3.1% APTB prevalence and 51.5% LTBI prevalence in low and middle-income countries (Fox et al., 2013). It mainly depends on host immune and inflammatory responses along with genetic makeup, which are essential for protection against *M.tb* (Aravindan, 2019; Rapolu et al., 2021).

Several studies suggested that polymorphisms in cytokine genes are associated with TB disease and may enhance understanding of the pathogenesis of TB (Aravindan, 2019; Wu et al., 2019). Th1 cells secrete interferon-gamma (IFN-γ), which plays an essential role in granuloma formation and the clearance of *M.tb*. The interaction between alveolar macrophages and *M.tb* is the initial event in the host-pathogen relationship, determining the infection outcome (Alamelu Raja, 2004). The effective activation of cell-mediated immunity is essential for establishing a protective immune response against *M.tb*. IFN-γ is critical for initiating immune responses and providing protection, as the innate immune system is rapidly activated, while the adaptive immune system works synergistically to prevent mycobacterial growth and spread (Khan et al., 2016).

IFN-γ is an essential cytokine in immunopathogenesis of TB and has been subject to several polymorphisms studies for pulmonary tuberculosis susceptibility(Abhimanyu et al., 2013).

IFN-γ rs1861494 A/G positioned on the third intron within the regulatory region of chromosome 12q15.(Rolandelli et al., 2018)**.** Until now, no studies have been conducted in the South Indian population analyzing the association between an IFN- γ rs1861494 and APTB. In the present study, we investigated the role of IFN-γ gene +2019A/G (rs1861494) polymorphism to identify the associations with TB. The APTB patients, along with their HHCs, are to predict the TB risk.

Materials and methods:

The present study involved the recruitment of N=331 participants, comprising N=102 individuals diagnosed with APTB, N= 123 their HHC's and N=106 age gender matched HC without any personal and family history of TB. The APTB patients and their HHC's, who attended the free chest clinic, Tuberculosis Unit (TU), PPM-DOTS (Public Private Mix-Directly Observed Treatment Short-Course) under the RNTCP (Revised National Tuberculosis Control Programme) implemented at Bhagwan Mahavir Medical Research Center (BMMRC) were taken into the study. APTB patients were confirmed and categorized based on sputum culture for *M.tb*, sputum microscopy for Acid Fast Bacilli (AFB) and Chest X-ray as per RNTCP guidelines. The mean ages for APTB cases, HHC, and HC were 31.38 ± 12.93 years, 30.32 ± 11.75 years, and 34.17 ± 11.34 years, respectively. Informed written consent was taken from the study subjects, who are willing to take part in the study were included and subjects, who are unwilling to take part in the study and with history of HIV, diabetes, malignancy, cardiac disease, immunodeficiency were excluded.

The study design was reviewed and approved by the Institutional Ethics Committee (IEC), Institute of Genetics & Hospital for Genetic Diseases (IGHGD), Osmania University and all the participants were explained about the study and informed consent was acquired from the subjects.

Sample collection:

A 2ml sample of venous blood was collected from study participants into EDTA coated vacutainer tube. DNA was isolated and stored in cryo-vials at -20°C for further use.

DNA Isolation:

Genomic DNA was isolated from whole blood using commercially available Flexi gene (Qiagen) kit by following manufacturer's manual. The purity of DNA and its concentration was measured using spectrophotometric instrument at 260 nm and 280 nm wavelengths, and were further confirmed by 1% agarose gel electrophoresis.

Genotyping:

Genotyping for the IFN- **γ** +2019A/G (rs1861494) cytokine gene was carried out by ARMS PCR (Amplification Refractory Mutation System Polymerase Chain Reaction) method.

Amplification was done by following a set of primers (100 pmol/µl). Based on the literature assistance primers were selected for IFN- **γ** gene and genotyping was carried out using ARMS.

Primers:

Sequence of primers used were as follows:

Allele A specific reverse: 5' AAGTAGGTGAGGAAGAAGCA **3',**

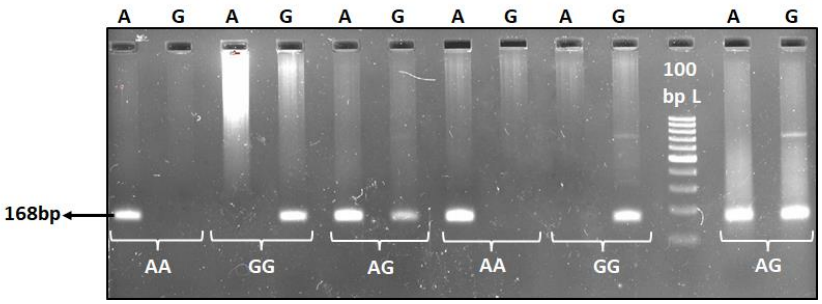
Allele G specific reverse: 5' AAGTAGGTGAG GAAGAAGCG **3',**

Common forward 5′-CCTTGGTGGCTGAGTTGG-**3′.**

Concentrations and cycling conditions for each PCR reaction was performed in a volume of 10 µl, consisting of genomic DNA (100 ng), Forward primer 0.2 µl and reverse primers 0.2 µl, dNTPs (2.5 mM) (GeNei, Bengaluru India), reaction buffer (1X), and Taq DNA polymerase (1.5 U) (GeNei, Bengaluru, India).

PCR reaction includes an initial denaturation for 5 minutes at 95°C, followed by 31 cycles denaturation for 30 seconds at 94°C, annealing for 50 seconds at 62°C, extension for 45 seconds at 72°C and a final extension for 5 min at 72°C. The final amplified products were electrophoresed on a 2% agarose gel at 100 volts in the presence of ethidium bromide. The bands were visualized under UV light using a Bio-Rad gel visualization system. Each sample was loaded into two separate lanes, and a product size of 168 base pairs was observed (**Figure 1**). To check the accuracy of the products, samples were selected for confirmation, and all results aligned with the initial findings.

Gel picture for IFN – γ +2019A/G gene Polymorphism:



Agarose gel image showing the ARMS PCR product of IFN – γ +2019A/G Lane 1-2,7-8 AA genotype,3-4,9-10 GG genotypes,5-6,12-13 AG genotype and Lane 11 is 100bp ladder.

Statistical analysis:

Hardy-Weinberg equilibrium (HWE) with χ^2-test was performed using "Michael H Court" online calculator to assess genotype deviation and association between the observed genotype frequency and expected genotype frequency in all the three categories. Demographic features were stated as mean ± standard deviation (SD) and the results were analyzed. The Genotype distribution and odds ratios (OR) were analyzed in various models including the dominant, codominant, over-dominant, and recessive genetic models, using SNPstats (Online tool). Our analyses considered two-tailed tests, with statistical significance defined as a p-value <0.05.

1. Results:

Genotype and Allele distribution of IFN- γ gene polymorphism rs1861494 A/G:

Model	Genotype	HCs N=106	APTB Patients N=102	HHCs N=123	APTB Patients Vs HCs		HHCs Vs HCs	
					OR 95% CI	P-value	OR 95% CI	P-value
Co-dominant	A/A	44 (41.5%)	13 (12.8%)	17 (13.8%)	1.00	<0.0001	1.00	<0.0001
	A/G	53 (50%)	79 (77.5%)	99 (80.5%)	**5.04 (2.48-10.26)**		**4.83 (2.52-9.28)**	
	G/G	9 (8.5%)	10 (9.8%)	7 (5.7%)	**3.76 (1.26-11.21)**		2.01 (0.65-6.26)	
Dominant	A/A	44 (41.5%)	13 (12.8%)	17 (13.8%)	1.00	<0.0001	1.00	<0.0001
	A/G-G/G	62 (58.5%)	89 (87.2%)	106 (86.2%)	**4.86 (2.42-9.77)**		**4.43 (2.33-8.40)**	
Recessive	A/A-A/G	97 (91.5%)	92 (90.2%)	116 (94.3%)	1.00	0.74	1.00	0.41
	G/G	9 (8.5%)	10 (9.8%)	7 (5.7%)	1.17 (0.46-3.01)		0.65 (0.23-1.81)	
Over-dominant	A/A-G/G	53 (50%)	23 (22.6%)	24 (19.5%)	1.00	<0.0001	1.00	<0.0001
	A/G	53 (50%)	79 (77.5%)	99 (80.5%)	**3.43 (1.88-6.26)**		**4.12 (2.29-7.41)**	
Allele frequency:								
Wild allele	A	141 (66.50)	105(51.48)	133 (54)	0.534 (0.352-0.809)	0.002	0.593 (0.398-0.882)	0.007
Variant allele	G	71(33.50)	99 (48.52)	113 (46)	**1.872 (1.236-2.838)**		**1.687 (1.134-2.513)**	
Hardy Weinberg Equilibrium: HCs p-value: 0.207731; patients p-value: 0.00000; HHCs p-value: 0.00000								

The genotype and allele frequencies of IFN- γ rs1861494 A/G polymorphism were analyzed using chi-square test, Hardy–Weinberg equilibrium (HWE) and represented the result as odds ratio (OR), 95% CI (confidence interval), and p-value for APTB patients, HHC's and HC. The genotype distribution results observed in HC were 41.5% AA, 50% AG, and 8.5% GG, in APTB patient's

was12.8% GG, 77.5% GA, and 9.8% AA and in HHC was 13.8% GG, 80.5% GA, and 5.7% AA. The "A' allele frequency is higher in healthy controls, and "G" allele frequency is higher in APTB patients. In HC, the genotype distribution deviated from HWE (χ^2 = 0.2077; p>0.05), In APTB patients (χ^2 = 30.895; p<0.05) and HHC (χ^2 = 47.35; p<0.05), it followed. When patients compared with healthy controls, genotype of dominant model A/G (p =0.00001, OR = 5.04, 95% CI = 2.48-10.26), G/G (p < 0.0001, OR 3.76, 95%CI =1.26-11.21) and genotype of co-dominant model AG-GG (p<0.0001, OR 4.86, 95%CI = 2.42 -9.77) were showing the significant susceptibility towards the disease and when HHCs compared with healthy controls, genotype of the dominant model A/G (p < 0.0001, OR 4.83, 95%CI = 2.52-9.28) and genotype of the co-dominant model AG-AA (p < 0.0001, OR 4.43, 95%CI = 2.33-8.40) were also showing strong susceptibility to the disease and G allele is showing a significant risk p<0.05 in both patients and HHCs when compared with HCs.

Discussion:

Tuberculosis (TB) is a chronic infectious disease. The **chronic nature** of TB **reflects** the complex interaction between the pathogen and the host (Areeshi et al., 2019). Genetic factors in the host are important contributors in developing a wide range of complex diseases. Polymorphisms in different genes can influence and modulate the immune responses towards the disease. All 90 % of LTBI infected individuals are not progressing to TB disease, but 10% of individuals with a weaker immune response might develop active TB disease (Aravindan, 2019). IFN-γ is a major Th1 type cytokine primarily produced by natural killer cells.(Schoenborn & Wilson, 2007). It plays a key role in macrophage activation and is essential for controlling *M.tb* infections. (Lee et al., 2015) The rs1861494 single nucleotide polymorphism (SNP) in the IFN-γ gene has been studied across various populations to assess its impact on TB susceptibility, and the results were inconsistent. A study from the North Indian population showed six novel associations of IFN-γ gene variants with susceptibility to PTB(Abhimanyu et al., 2012). Our results demonstrated that AG and GG genotypes of IFN- γ+2019A/G polymorphism were associated with an increased risk of TB in APTB patients and their household contacts. Our findings were correlated with a study that demonstrated that the GG genotype confers the risk of TB in Han Taiwanese (Lee et al., 2015). In contrast, A study from Argentina population showed that IFN- γ +2019A/G polymorphism was associated with tuberculosis resistance in a dominant model (Rolandelli et al., 2018). Besides, two studies showed no association between the IFN-γ +2019A/G polymorphism and tuberculosis in Croatian population the Chinese population (Etokebe et al., 2006; Yang et al., 2013).

This study supports the hypothesis that polymorphisms in IFN- γ +2019A/G gene influence the susceptibility to TB. However, due to the limited studies and small sample size of this study, our findings could be considered preliminary work, which needs to be confirmed in a larger sample size in future studies.

Author's contribution:

Bhagya Laxmi Rapolu: Performed the experiments, data analysis, writing, editing, and revising the manuscript; **Ashwini Pullagurla:** Performed the experiments: **Divya Aitharaju:** writing; **Pardhanandana Reddy Penagaluru:** Valuable inputs; **Suman Latha Gaddam:** Supervision, conceptualization, resources, and revising the manuscript. All authors read and approved the final manuscript.

Conflict of Interest:

The authors declared that they have no known competing interests for the work reported in this paper.

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