Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 5099 - 5111
Received 05 March 2021; Accepted 01 April 2021.

Role of IFNγ+874 AT gene Polymorphism in the Susceptibility of Pulmonary Tuberculosis in an Iraqi Patients

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ABSTRACT

Background: Cellular immune immunity mediates Host immune action against Mycobacterium tuberculosis, which is critical to cytokine and Th1 cells. TNF-alpha appears to be primordial in controlling infection with mycobacteria. This cytokine acts in Synergy with IFN-gamma, stimulates development of intermediate reactive nitrogen (RNI) media that mediate macrophage tuberculostatic function and stimulates immune cell migration to the infection site and helps in the formation of granuloma that regulate the progression of disease. IFN-Gamma is the principal cytokine involved in mycobacterial immune response, and its primary function is to activate macrophages to allow them to perform their microbicidal role. In comparison to TNF-alpha and IFN-gamma, IL-10 is primarily considered an inhibitory cytokine which is essential to the proper balance of inflammatory and immunopathological responses. The increase in IL-10 levels appears to help the host mycobacteria's survival.**Methods:**Study population included patients with pulmonary or extrapulmonary tuberculosis from Babil province - Iraq. Whole blood samples were collected from 67 TB cases at consultant clinic for respiratory diseases in Hillah - Babil province in the period between October 2020 to June 2021. Out of the (67) TB patients, there were (30) male and (37) female. The patients age range was from 1 to 80 years. The inclusion criteria used in this study was that all the TB cases must meet the clinical diagnosis criteria and confirmed by at least one of the laboratory tests (Smear, PCR and culture). The clinical diagnosis was conducted by the specialists in the consultant clinic for respiratory diseases. While apparently healthy persons included (39) persons equal (24) males and (15) females with age range approximately matched to that of patients. As pathogenesis of TB and associated with IFNγ+874 AT has a hereditary foundation, primer investigation was intended to evaluate the effect on the TB and Healthy people. Genotyping was performed utilizing single stranded polymorphism-polymerase chain response (SSP-PCR).**Results**his study revealed that IFNγ+874 AT genotype was significantly associated with susceptibility to tuberculosis in patients.it increased the risk of TB, in addition, IFNγ+874AT genotype in the control group in this study, had a higher frequency than AA, TT. This indicates that it has a protective effect against tuberculosis

Keywords: IFNγ+874 AT, Tuberculosis, Hillah

Introduction

The world's biggest health problem remains Tuberculosis, caused by Mycobacterium tuberculosis (Mtb) [1]. This is mainly due to the pathogen's ability to subvert host immunity, the

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recurrent existence of this disease, the inability of the present drug regime to fully eradicate the bacillary burden and drug-resistant strains [2,3]. Further clarification of the host defense mechanisms and the countermeasures employed by Mtb are critical for new approaches to reducing Mtb infection. A major method used to prevent an intracellular pathogen from spreading is to starve the bug of vital resources including amino acids. For example, host limits infections with Chlamydia and Leishmania by starvation from tryptophan amino acid. Since the tryptophan auxotrophics are normal, this strategy helps to eliminate infections [4,5]. However, by biosynthesizing tryptophan de novo Mtb evades this form of host immune strategy [6].

The interferon gamma release assay (IGRA) is a commercially available cost-effective assay that detects changes in interferon gamma (IFN-γ) caused by MTB infection. The WHO guidelines [7] rejected the recommendation of the IGRA for differentiating active TB, especially in high-burden countries, but the guidelines published by the European Centre for Disease Prevention and Control (ECDC) proposed that the IGRA could contribute supplementary information for patients who test negative for acid-fast bacilli (AFB) and MTB culture in sputum. In recent years, an increased number of studies have researched the utility of the IGRA to diagnose TPE [8].

While almost a third of the world's population is believed to be infected by M. tuberculosis, there are no clinical symptoms for a significant proportion of the population. But, as shown by cultivable sputum bacilli and other clinical symptoms [9], the remaining 5–15 percent of the population is affected by active disease. This shows that besides the mycobacteria themselves, the variations in the host susceptibility to TB can also be determined by host genetic factors [10]. In previous reports involving candidate genetic approaches and genetic association studies, several host genetics affecting susceptibility of a number of diseases have been identified [11–12]. The discovery of host genes and genetic differences, which play a major role in TB susceptibility and tolerance, will also help to explain the pathogenesis of this disease and could lead to new treatment and prophylaxis approaches. The protective immunity of the cytokines, their genes and receptor are impacted and pathological severity associated with altered circulatory levels of pro-inflammatory and down-regulatory cytokines in the case of TB [13] has also been affected. TB infection is generally known to be a protection reaction for macrophagen, and Th1-mediated immunity against M. tuberculosis. The human interferon α (IFNμ) gene is found on 12 (12q14) chromosome and contains four exons covering approximately 6 kB of exon. The main T-helper (type-1) cytokine formed by natural killer cells (NK) and T cells is Interferon μ (IFN-μ), encoded in IFN-т (also called IFNG). Innate immunity requires IFN-μ development and plays a key role in the activation of the macrophage to remove Mycobacterium infection [14].

An earlier study has shown that a malfunction in the IFN-α gene can lead to M. tuberculosis infection [15-16]. In the promoter or coding areas of cytokine genes, single nucleotide polymorphisms (SNPs) induce altered transcriptional functions as well as the differential secretion of cytokine. In addition, polymorphisms within the promoter or coding area genetically lead to Inter-individual differences in IFN-β development. The binding point for the NFkB

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transcription factor is an SNP located at +874 A>T (rs2430561) on the first intron of the IFN-Ţ gene, which putatively influences the gene expression and the secretion of cytokine, which significantly affects the results of infections. TB patients with the homozygoticallelel A combination have been reported to produce significantly less than single copies of allele T [17]. Variable linkages with TB susceptibility and severity also appear in this polymorphism [18]. These studies showed the significance of these cytokines for TB infection production and indicated that the variations in TB susceptibility could account for this genetic version of the IFN-β gene. In recent years, a number of case control studies have assessed the combination of the IFN-μ +874 A>T gene polymorphism with the expression of PTB in different populations after considering the functional importance of that genetic variant. Results in these studies have revealed mixed results and whether this polymorphism has been correlated with an increased or decreased PTB susceptibility [18-20] remains uncertain. The insufficient statistical capacity of each of the studies, including non-homogeneous people and individuals of different ethnic groups, is a likely reason to explain the noted contradictions in the results. In order to address this situation a statistical meta-analysis technique now is used to analyze the risks associated with the genetic condition, using a quantitative method to integrate the data from individual studies where sample sizes are too small to produce accurate conclusions [21].

Materials & Methods

Study Subjects (Patients & Control):

Study population included patients with pulmonary or extrapulmonary tuberculosis from Babil province - Iraq. Whole blood samples were collected from 67 TB cases at consultant clinic for respiratory diseases in Hillah - Babil province in the period between October 2020 to June 2021, out of the (67) TB patients, there were (30) male and (37) female. The patients age range was from 1 to 80 years. The inclusion criteria used in this study was that all the TB cases must meet the clinical diagnosis criteria and confirmed by at least one of the laboratory tests (Smear, PCR and culture). The clinical diagnosis was conducted by the specialists in the consultant clinic for respiratory diseases. While apparently healthy persons included (39) persons equal (24) males and (15) females with age range approximately matched to that of patients. As pathogenesis of TB and associated with IFNγ+874 AT has a hereditary foundation, primer investigation was intended to evaluate the effect on the TB and Healthy people. Genotyping was performed utilizing single stranded polymorphism-polymerase chain response (SSP-PCR).

Methods:

Blood Sampling:

About five milliliters of venous blood were collected from each patient in this study. The blood was divided into two parts: one part (about two milliliters) was collected into EDTA containing tubes for genetic part. The second part of the blood was placed in gel tube for thirty minutes, then transferred to plain tube and serum was obtained by centrifugation at 3000 rpm for 15 min;

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after that the serum collected and kept in the freezer (-20 ºC) until it was used for the immune and viral assay.

Isolation of genomic DNA:

Genomic DNA was used for molecular study by sequestered from the fresh blood, which collected in tubes of anti-coagulant EDTA and for frozen blood samples we recommended using protease K were applied using for DNA purification; Favor prep Blood genomic DNA purification kit. The isolation of DNA depended on the 5-stage procedure utilizing salting out techniques **[22,23]**:

- Lysis of the RBCs in the Cell Lysis Solution.
- Lysis of the WBCs and their nuclei in the Nuclei Lysis Solution.
- A salt out precipitation step using the Protein Precipitation Solution then removed the cellular proteins.
- The genomic DNA was concentrated and desalted by Isopropanol precipitation.
- The genomic DNA was rehydrated using the DNA Rehydration Solution.

The Estimation of DNA Concentration and Purity:

The DNA concentration of samples was estimated by using the Nano drop by putting 2.5µl of the extracted DNA in the machine to detect concentration in ng/µL and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ration for purifying DNA was between 1.7-1.9.

Electrophoresis of Agarose Gel:

Agarose gel electrophoresis was embraced to affirm the nearness and uprightness of the separated DNA after genomic DNA extraction **[23,24]**.

Gel Electrophoresis Reagents:

- Powder of Agarose
- TBE Buffer with 1X concentration
- Loading dye
- Safe Red
- DNA Ladder Marker

Protocol of Gel Electrophoresis:

Tris Borate EDTA Buffer preparation (1X TBE)

This solution was prepared by adding 900 ml Distill water to 100 ml 10X TBE (Promega/ Germany), forming 1 liter of (1x) TBE buffer **[23]**.

Preparation of agarose gel:

a) The amount of 1 X TBE (100 ml) was taken in a beaker
b) Agarose powder (1.5 gm) was added to the buffer
c) The solution was heated to boiling using a microwave oven for 2 min.
d) Safe Red (5 µl) of (10mg/ml) was added to the agarose solution.
e) The agarose was stirred in order to be mix and avoid making bubbles.
f) The solution was left to cool down at 50 – 60 C°.

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Casting of the horizontal agarose gel:

Subsequent to settling the brush in 1 cm a long way from one edge, the agarose game plan was filled the gel plate. The agarose was allowed to bond at room temperature for 30 minutes. The modified brush was absolutely removed and the gel plate was set in the gel tank. The tank was stacked with 1 X TBE support until it accomplished 1-2 mm over the surface of the gel.

DNA Loading & Electrophoresis:

DNA (3 µl) was mixed with (2 µl) loading dye. The samples loaded carefully into the individual wells of the gel, and then electrical power was turned on at 70 volts for 1 hour, afterwards the DNA moved from cathode (-) to anode (+) poles. The SAFE RED-stained bands in the gel were visualized using UV. Trans illuminator at 350 nm and photographed.

PCR protocol

A successful PCR program depends on the reaction conditions including reagents, temperature and the prevention of contamination. Previous study indicates that PCR is sensitive to reaction condition and that the optimization of these conditions is necessary to reach the highest specificity and product yield. Standard amplification conditions were applied in PCR with primer sequences. The annealing temperature in which primers hybridize to complementary sequences on the template DNA is perhaps the most critical in PCR programming. A master premix of Biolab was used, with components in table (1).

IFN-gamma (+874T/A) genotyping Genetic generation of IFN-c (+874T/A) with minor modifications was performed with refractory amplification mutation system-Polymerase chain reaction (ARMS-PCR) (8). The PCR response solution included 59 PCR buffer (s, 1.6 mM Mg2+), 0.2 mMdNTP (smaller and lower) dNTPs and 0.6 U/l Taq DNA Polymerase (Biolab Co., UK). In 25 lL of PCR reaction for genotyping, Allele A, including primary A and common primer P, as well as allele T, including primary T and common primer P were used.

The PCR conditions comprised an initial 95 degrees for 1 minute, and ten cycles each consisting of 15 s for 94 degrees Celsius, 50 s for 62 degrees Celsius, and 40 s for 72 degrees Celsius. The second denaturation period consisted of 30 cycles of 20 s at 95 °C, 50 s at 57 °C and 50 s at 72 °C respectively (Veriti, USA). Electrophoresis of 2 percent agarose gel was evaluated for PCR products, and a product dimension of 261-bp was identified.

Table 1. Sequence of primers

Gene	Primer	bp.	Ref.
IFN-ɣ (+874T/A)	5’-TTC TTA CAA CAC AAA ATC AAA TCA-3’	261	[25]
	5’-TTC TTA CAA CAC AAA ATC AAA TCT-3’		
	5’-TCA ACA AAG CTG ATA CTC CA-3’		

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The gradient condition for *IFN-ɣ (+874T/A)* are similar as shown in the following table (2). The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl of each forward and reverse primer in 20 µl of total reaction volume

Table 2. Gradient condition for *IFN-ɣ (+874T/A)*

Step	**Temperature C°**	**Time/min.**	**Cycles**
Initial denaturation	94	3 m	1
Denaturation	94	30 s	30
Annealing Zones	48-50-52-54-55-56	30 s	
Extension	72	40 s	
Final extension	72	7 m	1
Storage	4	∞	

After the determination of optimum annealing temperature for *IFN-ɣ (+874T/A)* by selecting the clearest band, which is 54 C°, PCR mixture was 5µl DNA, 5µl master mix, 5µl forward and reverse primer, PCR conditions were performed as in the following table (3).

PCR items were dissected on 2% agarose gel recolored with 5 µg/ml Safe red. The product was 261 bp.

Table 3. PCR condition for *IFN-ɣ (+874T/A)*

Step	**Temperature C°**	**Time/min.**	**Cycles**
Initial denaturation	94	3 m	1
Denaturation	94	30 s	30
Annealing	54	30 s	
Extension	72	40 s	
Final extension	72	7 m	1
Storage	4	∞	

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Results and Discussion

Susceptibility to tuberculosis and progression of the disease depend on interactions between the bacterial agent, host immune system, and environmental and genetic factors. In this case-controlled study, we aimed to determine the role of single-nucleotide polymorphisms of interferon-gamma, (IFN-ɣ +874 T/A) in susceptibility to tuberculosis [26]. The findings of ARMS-PCR revealed a 261-bp DNA fragment based on specific primers for A and T alleles. AT alleles was the most frequent genotype in both PTB (89.55%) and control (94.87) groups. The next two most frequent genotypes in TB patients were AA (5.97%) and TT (4.48%), respectively, in this study it found *IFNγ*+874 AT genotype was significantly associated with susceptibility to tuberculosis in patients.it increased the risk of TB. In addition, *IFNγ*+874AT genotype in the control group in this study, had a higher frequency than AA, TT. This indicates that it has a protective effect against tuberculosis. *IFN-γ* , which is a product mainly of natural killer (NK) and activated T cells, has an important role in host defense against mycobacteria. the main function of IFN-γ is macrophage activation, rendering them able to exert its microbicidal functions. It operates also by enhancing the antigen presentation through the induction of the expression of molecules from the MHC class I and II and promoting the differentiation of CD4+ T lymphocytes to the Th1 subpopulation [27]. The transcriptional regulation of the promoter region of the *IFNγ* gene is under the control of factors such as nuclear factor NF-κB(nuclear factor kappa- B), which binds to the 50 region of the human gene*IFNγ* and increases the expression of*IFNγ* [28]. Polymorphisms in the*IFNγ* gene at position +874 T/A led to genotype TT, TA or AA and might regulate cytokine production in susceptible individuals [29]. We found that the AT and AA genotype frequency was higher in TB patients and the TT genotype was significantly lower in TB patients. Statistical analysis showed that genotype AT causes fold increase in susceptibility to TB. The results of our study were consistent with those of previous studies in Taiwanese populations and populations of southeastern, Iran [30-31] In addition, the increased frequency of AT among TB patients in our study was consistent with several studies carried on Caucasian and Asian ethnicities in USA, India, Brazil, Taiwan and China [32-34].

Table 4. Genotype and allele frequency of *IFN-ɣ (+874T/A)* associated with Tuberculosis patients and control

IFN-ɣ (+874T/A)	**Patients (67)**		**Healthy (50)**		**Total**	
	No	%	No	%	No	%
Genotype						
AA	4	3.77	1	0.94	5	4.72
AT	60	56.60	37	34.91	97	91.51

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TT	3	2.83	1	0.94	4	3.77
total	67	63.21	39	36.79	106	100.00
Allele Frequency						
A	68	38.64	3	1.70	71	40.34
T	66	37.50	39	22.16	105	59.66
Total	134	76.14	42	23.86	176	100.00

In a study he come out Naz, et al,.2019 in a Pakistani population it was compatible with our study which showed the heterozygous genotype frequency (AT) did not significantly differ between the control subjects (34.17%) and TB patients (36.12%), *p*-Value= 0.628 [35]. These results were opposite with those found among Egyptian patients in a previous study, where the AA genotype was more frequent among TB patients [36]. Furthermore, other studies found a higher risk of TB in homozygous AA patients [37-38]. A study was carried out by [39] showed that the +874 A/A genotype was more frequent among patients with tuberculosis, and a significant association between the presence of the +874 (A) allele and active tuberculosis was observed.

The results of this study came were inconsistent with the results of other local study conducted in Babylon province by (Ahmed.,2019) which showed that the *IFN- ɣ* +874 A/A genotype was more frequent among patients with tuberculosis, (62.5%), The *IFN- ɣ* +874 A allele was significantly associated with increased susceptibility to tuberculosis. Individuals with two A alleles (Homozygous for the allele A) were significantly overrepresented among the patients with tuberculosis, 28 (46.6%), as compared with healthy control subjects, 3 (15%) and had a 4.958-folds increased risk of developing tuberculosis than the other two genotypes (AT and TT),

Two studies in the Chinese population came were inconsistent with our study which showed the *IFNG*+874AA genotype associated with risk (*P*<0.001 and *P*=0.039) [34]. In the Egyptian, South African and Tunisian populations, the *IFNG*+874AA genotype is also associated with risk of PTB+EPTB (*Pc*=0.015, *P*= 0.017, *Pc*=0.001, respectively) [40-41], In a study conducted in a sample control of Iranian population came consistent with our study that shown *IFNγ*+874AT genotype was the most frequent genotype in control subjects (66.9%, P value= 0.342) [30].

Some studies showed no association between IFN-γ +874T/A polymorphisms and vulnerability to tuberculosis [42, 43]. In addition, study conducted by others that shownnot Associated between polymorphisms IFNG+874T/A andsusceptibility to tuberculosis in both TB and control group [44].

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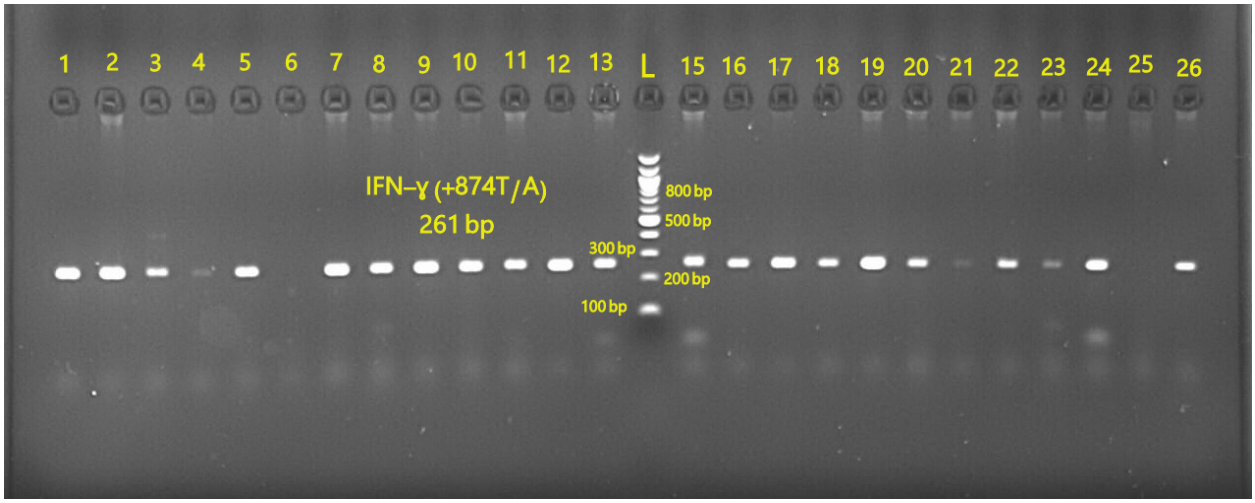

Figure 1. The Electrophoresis Pattern of *IFN-γ (+874T/A)*Polymorphisms.L lane contain the 100 bp DNA Ladder, 5 % NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl Safe red stain.
(1-5, 7-13) Lanes positive results for AA genotype
(15-20) Lanes positive results for AT genotype
(21-24, 26) Lanes positive results for TT genotype

ACKNOWLEDGMENT

Authors thank Hospital Staff of MERJAN for their help in specimen's collection. This study was supported by Faculty of Science for women and Department of biology at Babylon University.

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