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A Pilot Study of Genetic Polymorphism of Interleukin-10 and Interferon-γ Genes as Potential Susceptibility Factors in Tuberculous Egyptian Children

[1]Khalid Taman, [2]Howida M Sharaf, [3]Nahla M. Awad, [1]Howida Elgebally and [4]Manal Kishk

[1]Pediatrics Department, Institute of Post Graduate Childhood Studies, Ain Shams University;
[2]Clinical Pathology Department, Ain Shams University Hospitals;
[3]Pathology Department, Ain Shams University Hospitals and
[4]MS Pediatric Fever hospitals, Cairo, Egypt.

Abstract: Background: Tuberculosis (TB) is a world number one killer infectious disease. It has been estimated that 3.1 million children under 15 years of age are infected with TB worldwide. The risk of acquiring infection and developing severe complications are related to genetic variability at cytokines loci. Single nucleotide polymorphisms (SNPs) located at these genes could influence cytokine levels and regulate resistance and susceptibility to TB. Interferon-gamma (IFN-γ) is crucial to control intracellular growth of Mycobacterium tuberculosis while interleukin-10 (IL-10) has an antagonistic role. The aim of this study: was to assess the functional significance of single base change polymorphic variant identified in the promoter region of IL10 (G-1082A) and the first interon of interferon gamma (T+874A) genes and its relationship to TB susceptibility. Subject & methods: Forty Egyptian tuberculous children and 40 healthy controls were genotyped using Allelic discrimination assay of IL10 (G-1082A) and IFN-g (T+874A) genes polymorphisms in a pilot study. Results: IL10 – 1082 G and IFNγ + 874 A alleles frequency were higher in TB patient as compared to healthy control (67.5%vs 7.5% and 50%vs 15% respectively) with a significant association between TB and those alleles (p =0.000). Regarding relapse, and microscopy positive TB There was a statistically significant difference between IL-10 (A-1082G) genotypes (p=0.023 & p=0.009) meanwhile there was no statistically significant difference as regards INF-g +874 genotypes. Conclusion: IL-10 (A-1082G) and IFNγ (T+874A) genes polymorphism might be crucial for protective immune responses and may serve as biomarker of protection or susceptibility.

Key words: TB, Single nucleotide polymorphisms (SNPs), IL-10 (A-1082G) IFN-g (T+874A),

INTRODUCTION

Tuberculosis (T.B) has been declared as a major global health threat by World Health Organization (WHO) since 1993. About 9 million people develop TB annually, of whom 2 million die. The reported percentage of TB cases occurring in children varies from 3% to more than 25% (WHO, 2006).

Various studies correlate cytokine production with TB disease progress. Polymorphisms in the regulatory and intronic regions of several cytokines have been associated with different cytokine production (Polio *et al.*, 2002). Interferon gamma (IFNγ) is a key T helper (Th) type 1 cytokine Produced primarily by natural killer cells and T cells. Its production plays a pivotal role in macrophage activation for controlling M. tuberculosis infection (Collis and Kaufmann, 2001). Mice with a disrupted IFNγ gene when challenged with M. tuberculosis, fail to produce reactive nitrogen intermediates and restrict the growth of bacilli (Tso *et al.*, 2005). Human with inherited complete or partial IFNγ receptor deficiency are highly susceptible to infection by atypical mycobacteria (Tso *et al.*, 2005).

There is a single nucleotide polymorphism (SNP) + 874 (A/T) located at the 5 – end of a CA repeat at the first intron of human IFNγ. The + 874 T allele is linked to the 12 CA repeats, whereas the A allele is linked to the non – 12 CA repeats (Pravica *et al.*, 2000). The specific sequence of the T allele is found to provide a binding site for the transcription factor nuclear factor KB (NF- KB). As NF- KB induces IFNγ expression, this T allele correlates with high IFNγ expression whereas the A allele correlates with low expression. A part from + 874 (A/ T) two potentially functional polymorphisms have also been repeated at the promoter – 179

Corresponding Author: Nahla M. Awad, Pathology Department, Ain Shams University Hospitals, Cairo, Egypt. E-mail: dr.n.awad@gmail.com

(179 (6/T) and 3 untranslated region + 4766 (C/T) (Bream *et al.*, 2002).

IL10 is a plethoric cytokine with important immune- regulatory and immune-stimulatory functions. IL 10 can act as an anti- inflammatory cytokine and can enhance B cell proliferation, differentiation and immunoglobulin production (Th2 cytokine) (Polio *et al.*, 2002). The gene encoding IL 10 has been mapped to chromosome 1(Kim *et al.*, 1992). Three single base pair substitutions in IL 10 gene promoter at positions – 1082 G/A-819 T-C and 592 A-C from the transcriptional start site have been identified (Kube *et al.*, 1995 and Turner *et al.*, 1997). The polymorphism at position-1082 lies within an Ets (E- twenty six specific) like recognition site and may affect the binding of this transcriptional factor and therefore alter transcriptional activation; the- 1082 A allele correlates with low IL10 production after stimulation of T cells in vitro, while polymorphisms at positions -819 and- 592 do not seem to be involved in the differential production of IL 10(Yang *et al.*, 2007).

Accordingly, the aim of this work was to assess the functional significance of single base change polymorphic variant identified in the promoter region of IL10 (-1082 G /A) and the first interon of IFNγ (+874A/ T) genes and its relationship to TB susceptibility.

MATERIALS AND METHODS

This case- control study included forty TB children who were stationed at the Abbassia Chest hospital, as a pilot study in Egypt. Their age ranged from 5-15 years. Twenty apparently healthy age and sex matched children were recruited as controls. Control subjects had no history of tuberculosis and no evidence of prior tuberculosis noted on chest radiography. The study protocol was approved by the ethics committee of Institute of Postgraduate Childhood Studies Ain Shams University, and written informed consent was obtained from study participants.

TB was diagnosed on the basis of clinical presentation, radiographic, Ziehl-Neelsen positive smear and/or *in vitro* cultures using *tuberculosis specific growth medium and t*uberculin testing for all cases was performed by intradermal injection of 2 tuberculin units of purified protein derivative (PPD) manufactured by VACSERA composition Egypt and the resultant diameter of transverse induration was measured after 72hours. Excisional biopsy for histopathological examination and fine needle asprirations (FNA) were also done. Assessment of socioeconomic (SES) standard was carried out (Abd El-Aziz and Shaks, 1995).

Fine needle aspirations were performed under sterile aseptic conditions on the suspected lymph nodes using sterile disposable 23 gauge needles and 10-cc syringes. A minimum of two smears for cytology and one smear for Ziehl Neelsen stain were prepared. Smears for cytology were stained with the Papanicolaou staining method. Patients were diagnosed as having tubercular lymphadenopathy on Fine needle aspirations cytology (FNAC) when the aspirate had epitheloid cells with either caseation necrosis or Acid fast bacilliAFB present. Excisional biopsy of the lymph node for histopathological examination was carried out in case of negative results of FNA. Radiologically guided transthoracic (percutaneous) FNAC has been required for diagnosis of pulmonary tuberculosis in case of failing of non invasive techniques (i.e., negative ZN smears) using 22-gauge needle.

Analysis of IL-10 and IFN γ Genes Polymorphisms:

IL-10 and IFN γ genes polymorphisms were studied by real–time PCR allelic discrimination (AD) assay on Applied Biosystem (ABI) 7300 fast real-time PCR system (Applied Biosystem, USA). AD methods permits the analysis of thousands of samples per day with high sample to sample reproducibility however other methods of genotyping as restriction fragment length polymorphism (RFLP) provide insufficient discrimination among isolates.

Allelic discrimination was performed by the use of specific probes for each allele respectively labeled with fluorescent reporter dye VIC and FAM. The presence of two primer/ probe pairs in each reaction allows genotyping of two possible variants at a SNP site in a target template sequence using TaqMan probe- based sequence detection chemistry and Reagent configuration.

For IL-10 and IFN γ polymorphism, a mix containing specific primers and fluorescent probes designed by Applied BioSystems called TaqMan SNP genotyping assay was used. It contains IFN- γ mutation + 874 (A/T) primers and probes with FAM dye and the wild type with VIC dye (Assay ID c_61106456_10) and IL-10 -1082 (G/A) mutation primers & probes with VIC dye and wild type with FAM dye (Assay ID c_31404405_10) obtained from Gene Bank (NM-005534,3-NM000572,2 respectively) and checked through the web with "Basic local Alignment search tool" programmed (blast F, ncbi home page, http://ncbi nlm.nih.gov/blast).

Genomic DNA was isolated from whole blood using QIAamp DNA Minikit (Qiagen, Germany) according to manufacturer's instructions and stored at-80 °C until used.

DNA amplification was performed with forward 5'cgaggcctgttgtctaccaagtgcagttta3' and reverse 5'gagtgtgacttcactgccgccagtcc3' primers of IFN γ (A/T)transversion susbstitution and forward 5'catcaaaaagaccgcatttcagttattt3' and reverse` 5'cttgctggaggactttaaggtgaga3' primers of IL10 (G/A) transversion susbstitution and AD was performed by the use of specific probes: IFN γ (VIC/FAM) 5'tcgtggtttgcgatccctctccccatagggtctgt ca ccctggggctgag3' and IL10 (VIC/FAM) 5'atccgattttggagacctctaattttgtcctagagtctatagagtcgcca 3'.

Some quality control (QC) blinded samples were distributed throughout the runs and genotyped for concordance of results and No template control (control samples without DNA samples- NTC) were systematically included in each genotyping plate.

Five microns of diluted purified genomic DNA (1:20) was pipetted to 13.75 µl of reaction mix (12.5µl/reaction of 2 X TaqMan universal PCR master Mix, NOAmp Erase UNG and 1.25 µl/reaction of 20 X SNP Genotyping assay mix) into reaction plate which was kept on ice until loading in ABI 7300 system. A pre-read run record the background fluorescence of each well of the AD plate document before PCR. During the post-read run, the pre-read fluorescence is subtracted from the post-read fluorescence to account for pre-amplification background fluorescence. Fluorescence data was analyzed automatically by 7300 fast SDS software which plot the results of AD run on scatter plate of allele X versus allele Y. The higher fluorescence should be homozygote and if equal fluorescence should be heterozygote.

Statistical Analysis:

Analysis of data was performed using SPSS version 13. Descriptive Statistics of all data were in form of mean ±SD for quantitative variables and in form of frequency and percentage (%) for qualitative variables. Chi-square test ($\times^2$), Odd ratio (OR) and confidence interval (CI) where applied for comparison of qualitative variables. Also Pearson correlation test was performed, values of $P < 0.05$ were considered statistically significant.

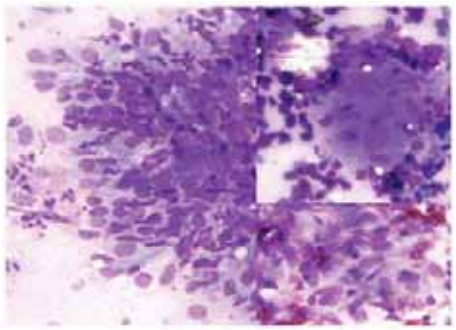


Fig. 1: FNA, Tuberculous lymphadenopathy showing epithelioid non caseating granuloma of early TB, Papanicolaou staining (X400).

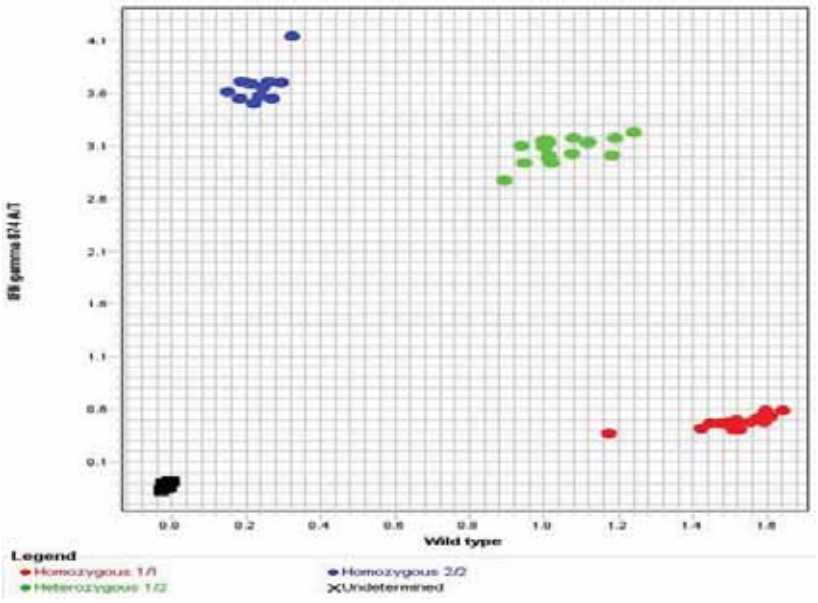
RESULTS AND DICUSSION

The demographic characteristics of patients with TB and healthy controls were given in Table (1).There was a statistically significant difference of socio-economic state (SES) and past history between patients and control groups (p=<0.0001and <0.0005 respectively). The patient groups included 28(70%) pulmonary tuberculosis, 6 (15%) TB meningitis, 5 (12.5%) TB lymphadenitis and one (2.5%) case with miliary TB.

IL10-1082 and IFNγ + 874 Alleles and genotypes frequency were shown in Table (2). IL10 – 1082 G and IFNγ + 874 A alleles frequencies were higher in TB patient as compared to healthy control (67.5%vs 7.5% and 50%vs 15% respectively). Thus a significant association was found between TB and those alleles {p =0.000, OR25.6, 95%CI (9.8-66.5)}&{(P= 0.000 OR=5.6, 95%CI (2.6- 12)} respectively. Genotypes analysis revealed 3 different genotypes AA, GA & GG of IL-10(-1082G/ A) and TT, AT& AA of IFNγ (+874A/ T) among patients however in control only AA genotype of IL 10 -1082 (90%) and AT (20%) and TT (75%) of IFNγ+874.

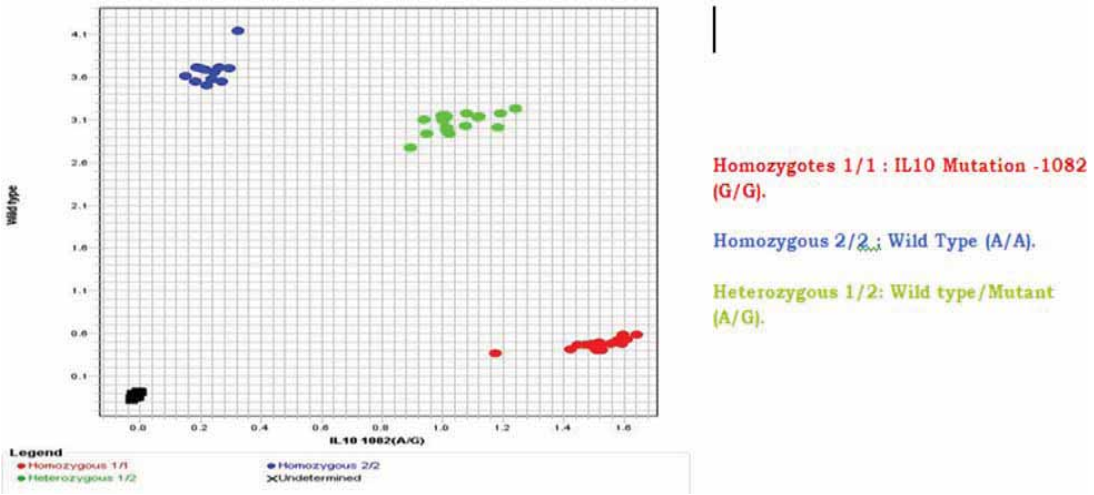
IL10-1082 and IFNγ + 874 Allele and genotype frequencies were analyses in TB relapse and smear microscopic examination as illustrated in table (3). There was a statistically significant difference of IL-10 (-

1082G/ A) genotypes between patients in relapse versus non relapse (p=0.023)with increase the frequency of GG genotype among patients in non relapses (52%), meanwhile there was no statistically significant difference of INF-g +874 genotype in relapse vs non relapse. There was a statistically significant difference in genotypes frequency of IL-10(-1082G/ A) in microscopy positive TB on comparing to microscopy negative. AA genotype was decreased in microscopically positive patients (9.1%) and increased in microscopic negative (57.1%). There was no statistically significant difference as regard IFNγ+874 genotypes.



Homozygous 2/2: Wild Type (T/T); Homozygous 1/1: Mutation IFN Gamma +874(A/A); Heterozygous 1/2: Wild type/Mutant (A/T).

Fig. 2: Allele discrimination plot of INF-g (SNP Assay: SNP Assay 2).



Homozygotes 1/1 : IL10 Mutation -1082 (G/G); Homozygous 2/2 : Wild Type (A/A); Heterozygous 1/2: Wild type/Mutant (A/G).

Fig. 3: Allele discrimination plot of IL-10 (SNP assay: SNP Assay 1).

Table 1: Demographic characteristics of the patients & healthy controls.

Demographic Feature	TB patients (N = 40)		Healthy control (N = 40)		X^2	P
Age (year) *	13.6± 1.78		13.5± 1.76		0.21	0.84
Sex (Female/ Male)	26(65%)/14(35%)		28(70%)/12(30%)		2.35	0.12
Socio- economic (SES)						
Very low	6	(15%)	32	(80%)		
Low	6	(15%)	6	(15%)		<0.0001
below average	26	(65%)	0	(0%)	28.36	
Above average	2	(5%)	2	(5%)		
Past history**						0.0005
+ve	17	(42.5%)	0	(0%)		
-ve	23	(57.5%)	40	(100%)		

*independent samples test; ** Fisher exact test

Table 2: Variant alleles and genotypes frequency of IL 10 (A–1082 G) and IFN γ (T+874 A) gene in TB patients and Healthy control.

IL-10-(-1082G/A) allele/ genotypes	TB patients N=40(%)	Control N=40(%)	P value	IFN-g (+874A/T) (allele/ genotypes)	TB patients N= 40 N (%)	Control N=40 N (%)	p-value
A	26(32.5)	74 (92.5)	0.000**(HS)	A	40(50)	12(15)	0.000** (H.S.)
G	54(67.5)	6 (7.5)		T	40(50)	68 (85)	
AA	4(10)	36 (90)	0.000**(HS)	AA	12(30)	2(5)	0.004(S)*
GA	18(45)	2 (5)		AT	16(40)	8(20)	
GG	18(45)	2(5)		TT	12(30)	30(75)	
OR, (95% CI)	25.6(9.8-66.5)			5.6 (2.6- 12)			

**HS highly significant * S: Significant

Table 3: IL-10 (-1082G/A) and IFN-g (+874A/T) genotypes frequency in TB relapse and smear microscopic examination in TB PATIENTS.

Variable	IL-10 (-1082 G/ A)					IFN-g (+874A/T)				
N	AA	GA	GG	χ^2	P	AA	AT	TT	χ^2	P
Relapse patients (N=15)	4(26.7)	6(40)	5(33.3)	7.53	0.023*S	5(33.3)	5(33.3)	5(33.3)	0.44	0.8
Non relapse patients (N=25)	0(0)	12(48)	13(52)			7(28)	11(44)	7(28)		
Smear +ve (N=33)	3(9.1)	14(42.4)	16(48.5)	9.35	0.009*S	12 (36.4)	13 (39.4)	8 (24.2)	2.34	0.31
Smear -ve (N=7)	4(57.1)	1(14.3)	2(28.6)			4 (57.1)	3 (42.9)	0 (0)		

*S: Significant

Dicussion:

TB in Egypt is considered an important public health problem, Egypt is ranked among the mid – level incidence countries with estimated incidence 25-29 per 100.000 population , TB mortality rate was 3 per 100.000 and case detection rate 60% which still below WHO requirement according to WHO report 2007global TB control (WHO, 2008).

This was the first study demonstrating the association of IL10 (-1082) and IFNγ (+ 874) genes polymorphism in TB among Egyptian children. TB hinders socioeconomic development: 75% of people with TB are within the economically productive age group of 15-54 years. Ninety-five percent of all cases and 99% of deaths occur in developing countries (Kim *et al.*, 1992). Most risk factors for the acquisition of TB are usually exogenous to the patient. Thus, the likelihood of being infected depends on the environment and characteristics of the index case. However, the development of active disease also depends on the inherent immunologic status of the host (Kube *et al.*, 1995).

This study revealed a highly statistical significant difference between TB patients and controls as regards socioeconomic state and past history. Similarly, WHO (2006) and Alcaiis *et al.,* (2006) confirmed the relationship between poor socioeconomic status and increased risk of TB. There were no statistically significant difference between IL-10 (A-1082G) and IFN-g (T+874A) genotypes and different clinical forms of TB patients. Similar results were reported by Casanova *et al.* (2004).

The 5´flanking region of the IL-10 gene is highly polymorphic with single base pair substitution at position-1082 G/A which result in differential IL-10 production. The G allele at – 1082 and haplotypes containing this allele have been associated with high IL-10 production while the A allele and the ATA haplotype have been associated with low IL-10 production (Turner *et al.*, 1997).

There was a significant association between IL-10 -1082G allele and TB in our study while no significant association was found between different clinical forms of TB and IL-10 (A-1082G) gene polymorphism. Although several studies examined the association of IL 10 promotor polymorphisms in TB susceptibility and severity. Discrepancies were observed among these studies. In some studies, IL-10 -1082 A allele and

heterozygosity for IL-10-1082 polymorphism were associated with TB susceptibility (Yang *et al.*, 2007 and Delgado *et al.*, 2002), while in other studies with large sample sizes, IL10 – 1082 A>G polymorphism were not associated with TB (Bellamy *et al.*, 1998 and Shin *et al.*, 2005). Two Turkish studies showed that IL-10 -1082 G allele frequency was found significantly more common in TB patients than healthy controls (Oral *et al.*, 2006) which are similar to our results. Both those studies and our study were performed with relatively small sample size. One possible explanation for the apparent inconsistency between these studies is that ethnic-specific genetic variation could greatly influence host immunity to tuberculosis , causing different tuberculosis susceptibility on the ethnic population studied .Another possible explanation might be involvement of more distal promoter element of IL-10 gene. Also, the relatively small size studied population may play a role causing variation.

In this study there was a statistically significant difference between different genotypes of IL10-1082 in patients as regards relapse versus non relapse and microscopy positive versus negative results. The increased frequency of AA genotypes in microscopy negative patients suggest that potentially low IL10 sector phenotype is associated with diminished bacterial replication indicating probable influence on modifying disease. Oral *et al.* (2006) reported that IL -10 inhibits monocyte/macrophage function during inflammation by down regulation the production of pro-inflammatory cytokines, such as IL 12 and TNF-α and suppressing the surface expression of major histocompatibility class II. IL-10 can also inhibit CD4 T cell chemotaxis towards IL-8 and T cell apoptosis by leading to Bcl-2 up-regulation. Interestingly, it can induce the proliferation of CD8+ T cell (Redpath *et al.*, 2001). The suppressor effect of IL – 10 on T cells has been recently shown to be directed to block CD 28 signaling cascade and subsequently phosphatidylinositol 3 kinas activation in T cell (Akdis *et al.*, 2000). Since modulation of T cell response by IL – 10 seems to influence the susceptibility of the host to TB infection (Fortschand and Rollinhoff, 2000), determining the polymorphisms in IL-10 genes may be beneficial for predicting the disease susceptibility.

This study results revealed that those carrying IFNγ+ 874 AA and TA genotypes had an increased risk of developing TB in our population as UK population (Pravica *et al.*, 2000). Bulat- Kardum *et al.* (2006) reported that IFNγ (+ 874) AA genotype is thought to provide a low secretor phenotype of IFNγ conversely the T/T + 874 genotype is supposed to make a high secretor phenotype. Moreover, Sallakci *et al.* (2007) reported that A allele was associated with lower IFNγ level which may impair the activation of macrophages resulting in TB development. Reduced IFNγ production and mRNA expression are observed from stimulated peripheral blood mononuclear cell (PBMC) in TB patients. This may be ascribed to the + 874 A/A genotype (Lopez *et al.*, 2003) and also the reduced expression of nuclear cyclic adenosine 5′-monophosphate response element- binding protein and reduced IFNγ promoter activity in TB patients. Moreover IFNγ production from PBMC is depressed in TB patients with A/A genotype when compared with A/T or TT genotype at the time of diagnosis and after completion of treatment. This suggests that A/A genotype may be related to TB severity or reactivation. Results of Lio *et al.* (2002), indicate an association between reduced +874 TT IFNγ homozygous frequencies and chronic lung TB suggesting a genetically determined high IFNγ production capacity is associated with resistance to chronic lung tuberculosis.

In the populations of Sicily, Spain, South Africa (colored), and Hong Kong Chinese, a positive association of IFNγ +874 (A/T) with risk of tuberculosis was observed (Lopez *et al.*, 2003; Rossouw *et al.*, 2003 and Henao *et al.*, 2005), but in, African Americans, Caucasians, Hispanics and West Africans populations, no association was observed as regard, polymorphism of the IFNγ gene and risk of tuberculosis (Lio *et al.*, 2002; Moran *et al.*, 2007 and Cooke *et al.*, 2006).

Furthermore, there is no direct evidence that this polymorphism causes a decrease in immune response specifically to M. tuberculosis. Thus, it can be seen that the + 874 A/T gene polymorphism may be one of the factors influencing disease susceptibility, and other factors or defects in different steps of the immune response (even other polymorphisms of IFNG or IFNGR) might also be responsible for an inability to prevent progression of TB infection to disease. However no significant association was found between different IFNγ (+ 874) genotypes and the development of relapse or microscopy positive / negative form of disease in this study. Etokebe *et al.* (2006) reported that the frequency of TT (+874) homozygote was significantly decreased in microscopy positive patients perhaps lower IFNγ secretion inferred from the lack of T at (+874) might influence replication of MTB thus influencing the outcome of this diagnostic procedure. Whether or not may confer increased risk of severe disease remains to be elucidated in another study. However, Yang *et al.* (2007) and Selvaraj *et al.* (2008) stated that no association was found between IFNγ +874gene polymorphism and disease susceptibility. Also Selvaraj *et al.* (2008) reported that weak association was found in IFNγ and IL-10 genotypes with the development of relapse. Difference in results between presented study and another could be due to different heplotypes structure with specific ethnic groups. Moreover, Selvarag *et al.* (2008)[32]

reported that lack of association between IFNγ level and IFNγ+ 874 polymorphism may not influence IFNγ response and probably human leukocyte antigen and other closely linked genes might have a role in influencing IFNγ + 874 level at least in south Indian population.

Etokebe *et al.* (2006) stated that some alleles affecting the expression level of IFNγ or their binding capacity might still be important for susceptibility to tuberculosis but they might be infrequent and thus below detection. Alternatively, the members of IL12/ IFNγ group which are functionally interconnect represent rate limiting step as a whole for immunity against MTB. Therefore, in certain individual the actions of hypomorphic alleles might be rescued by the actions of hypermorphic alleles that regulate the expression or action of the former gene. If that was the case then it would be difficult to observe separately any allele contribution to disease susceptibility.

The majority of TB patients included in this study had pulmonary TB. Therefore it was not possible to demonstrate an association between the system involved and the +874 A/T gene polymorphism. However, the paucity of patients with extra pulmonary TB limits statistical analysis of these data for a possible association between organ preference and host + 874 A/T allele. Therefore, from the course of this study, we can suggest that +874 AA/AT genotype might be a risk factor in the susceptibility for tuberculosis, especially for pulmonary tuberculosis in Egyptian population.

Bonecini *et al.* (2004) stated that there is cross regularity relationship between the two cytokines especially noted at therapy where there is decrease IL10 expression and increase IFNγ expression. Sahiratmadja *et al.* (2007) explained that there was suppression of IFNγ expression associated with increased level of IL-10.

Our findings suggest that IL-10 (A-1082G) and IFNγ (T+874A) genes polymorphism might be crucial for protective immune responses and may serve as biomarker of protection or susceptibility of TB. Allelic discrimination by real time PCR was of great value in detection of different genes polymorphism. Because the present study was performed with relatively small sample size, further studies with larger sample sizes are necessary to clearly elucidate the discrepancies and to investigate more potentially influential and polymorphic immune regulatory genes in patients with TB.

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