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Original Research Article

Polymorphism of Interferon Gamma Promoter and Receptor among Tuberculosis Patients in Basra Province, South of Iraq

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Tuberculosis again is about to be as nightmare that troubles slumber of the most advanced societies furthermore developing and non-developed societies. The main goal of the present study was to determine the correlation between the polymorphism in the promotor region of IFN-γ gene and IFN-γ receptor with TB outcome. Seventy-four cases were collected in the Institute of the Tuberculosis and Chest Disease – Basra city as confirmed cases with TB. Genomic DNA was extracted from 74 patients and equal number of healthy persons as control. IFN-γ promotor and *IFN-γ* receptor were amplified by conventional PCR by using two sets of specific primers. As result, there were nine single nucleotide polymorphisms (SNPs) found in IFN-γ promotor of patients but were not found control. It appeared that (G→T) SNPs were the most common SNP in patients with a percentage of (33.5%) of all SNPs, followed by (G→C) SNPs and (G→A) each SNP with a percentage of (19%). For Interferon gamma receptor1, the results showed there was relationship between the number of CA motifs and susceptibility to infection with TB. Only eight microsatellites were recorded in the patients. The allele $(CA)_{12}$ had no effect on susceptibility with TB infection, while the individuals which had $(CA)_{17}$ and $(CA)_{22}$ cleared to be the most apt to infection with (TB), but the alleles $(CA)_{13}$, $(CA)_{14}$ and $(CA)_{19}$ repeats were responsible for protective against tuberculosis. The mean IFN-g serum level was significantly depressed in Patients (26.92±5.29 pg./ ml), compared with healthy control (28.40 ±10.73pg/ml). From current study, we concluded that there is a genetic defect in IFN-γ genes was came from present of SNPs and variation in (CA) repeats the mater which had made some people more susceptible to infection than others with TB, this clarify re-outbreaks of infection from time to time. We strongly recommended doing additional study on other genetic factors that lead to increasing the susceptibility to tuberculosis disease in Iraqi community.

Keywords: Interferon Gamma Polymorphism, Tuberculosis/genetics, Single nucleotide polymorphism.

INTRODUCTION

The battle with tuberculosis disease, unfortunately, did not end. Tuberculosis is back once again to become a serious problem in developing and developed countries. *Mycobacterium tuberculosis* is the extensive cause of morbidity and mortality in the world [1]; Among infectious diseases, tuberculosis comes after AIDS as a main causative of death. Socialize can increase ability of the disease to spread because it primarily passed from person to another by inhalation aerosol droplets which thrown by infected individuals.

Infectious dose with MTB is 1-200, while the aerosol droplet may be contain 1-400 bacilli, so contact without infection about to be impossible. Furthermore, to its airborne infection, MTB is deft at employing the natural defenses of the host for its own advantage. Living in or having visited a high incidence area are the most important risk factors, but host factors also have a role in the risk of developing active disease. The period from which a person is infected by MTB up to the development of active disease different from person to person. There is no doubt that tuberculosis is a disease of poverty, also environmental and socio-economic factors have it's a role in infection, but only 10% of those infected individuals by MTB exhibit clinical symptoms of the disease, also the severity of the disease varies from person to person [2].

Familial clustering data, animal models, twin studies and complex segregation analysis and many others studies clearly demonstrated that there is a genetic basis to accept the incidence of tuberculosis. [3,4,5,6]. Determination the host genes, which are responsible for susceptibility and resistance to TB, may lead to better understanding of the pathogenesis of MTB also it may has a significant role in the prevention of tuberculosis which in turn could lead to development of more

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effective treatment and vaccines [7]. Inflammatory response stage is the second step after the cellular up taking of TB pathogen, in this step, pro- and anti-inflammatory cytokines and chemokines are produced. From these cytokines, interferon gamma (IFN-γ) which is the most important cytokines, it belong to type II family. IFN-γ is primarily produced by Th1 (CD4 and CD8), cytotoxic T lymphocyte (CTL), effector T cells and from natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response [8].

IFN-γ has a critical role in protective responses against intracellular pathogens. IFN-γ has many functions in immune system. Functions include; increase antigen processing and activation of macrophages, inhibition of activation of Th2 cells, increase expression of MHC molecules on cell surface, leukocyte migration and stimulation of NK cells [9, 10]. The IFN-γ gene is made up of four exons, with three introns, located on chromosome 12q24 spans approximately 5.4 kb. Production of IFN-γ is genetically controlled; variations in the IFN-γ regulatory genomic regions like promotor have been related to infection with TB.

In the other hand, interferon gamma receptor (INFGR) which composed of INFGR1 and INFGR2, plays a vital role in ligand binding, signal transduction of IFN-γ. INFGR1 situated on the long arm of chromosome 6 at position 23.3 (6q23.3), encodes the ligand-binding chain (alpha) of the interferon-gamma receptor. It has been reported that the CA repeat microsatellite allele in the fifth intron (in INFGR1) is associated with expression level of this most important cytokine (IFN-γ). Defects in IFNGR1 have been mentioned as a cause of Mendelian susceptibility to mycobacterial disease, also as familial disseminated atypical mycobacterial infection. In the present study*, we aimed to* determine the possible correlation between the polymorphism in the promotor region of IFN-γ gene and IFN-γ receptor with IFN-γ level in patient and healthy control, at any amount this genetic variation influence on TB outcome in Basra Province, South of Iraq.

MATERIALS AND METHODS

Seventy-four patients and 74 individuals as control was enrolled in this study provided by Salman [11]. patients' data in table (1). Blood samples collected from each patient and control by vein puncture using disposable syringes. From this blood, 4 ml was put in plain tubes and allowed to clot at room temperature for 30 min., then centrifuged for 15 min. at 3000 rpm, the serum was separated in tubes and stored at -20˚C, and thawed immediately prior to estimate of IFN-γ level by using of Human IFN-γ ELISA kit (Takara Biomedical Technology. China). ELISA test was performed according to company instructions.

For DNA extraction, 2 ml of collected blood was put in EDTA tubes, Reliaprep blood DNA Miniprep kit (Promega, USA) was used For DNA extraction, the extraction steps were done according to company instructions. The extraction process was verified by characterization of genomic DNA bands in agarose gel electrophoresis by loading of 6µl DNA mixed with 3µl of bromophenol blue in the wells of the 1% agarose gel.

Two Sets of primers for PCR amplification of IFN-γ promotor and IFN-γ receptor in table (2). For PCR reaction, the following reaction mixture was used: 1.5µl of genomic DNA, 12.5 µl of Premix Taq v.2 plus dye (Takara Biomedical Technology. China), 0.5 µl $MgCl_2$, 0.5 µl of each primer (GeneScript Make Research Easy, China) and 9.5 µl of nuclease free water. PCR conditions for amplifying INF- γ were initial denaturation at 94˚C for 5 min., followed by 35 cycles consist of 1 min. at 94°C, 1 min. at 65.3°C and 1 min at 72°C with a final extension at 72°C for 5 min. The PCR conditions for amplifying INFR1 gene were initial denaturation at 95˚C for 15 min., followed by 35 cycles consist of 30 sec. at 95°C, 1 min. at 56°C and 1 min at 72°C with a final extension at 72°C for 7 min.

The amplified products were determined by electrophoresis on agarose gel containing 0.5 µg/ml Ethidium bromide. Before sequencing, PCR products were purified by Gel / PCR Extraction Kit (BIOMIGA Ezgene, China) according to the manufacturer's recommendations. All samples were sent to GeneScript company (GeneScript Make Research Easy, China) for sequencing. Two types of file had came back from the company, ABI and text file. DNA Dynamo software was used to analysis the data results. Multiple alignments for high quality sequences were done for with each other plus with reference sequence at GenBank were performed to find DNA polymorphism within sequences.

RESULTS

Primers which was used for amplification *IFN-γ* promotor region and *IFN-γ* receptor had succeeded to produce 863bp, 190bp long amplicon for promotor and receptor. DNA amplicons of visualized by agarose gel electrophoresis whilst product sizes were determined by comparison with marker as in figure (1-A, B). Direct PCR sequencing performed partial mapping of the promotor regions of IFN-γ gene. Sequencing approach allows determining the multiplicity of new SNPs. http://www.oege.org/software/hwe-mr-calc.shtml.com.

The results showed there are nine single nucleotide polymorphisms (SNPs) that found in *IFN-γ* promotor of patients but not control, these SNPs distributed into five kinds. Table (3) shows there are five main SNP types, their frequency according their positions on *IFN-γ* promotor and percentage of each SNP from total population SNPs (42 SNPs). Therefore, it clearly appeared that (G→T) SNPs were the most common with a percentage of (33.5%) of all SNPs, followed by (G→C) SNPs and (G→A) SNPs with a percentage of (19%) for each SNP.

Forty-two SNPs were recorded in promotor of (74) patients, (G→T) SNP at locus (-714) was the most frequency (table (4)). However, the else four SNPs were shared the same frequency but they were distributed along nine positions. As result, of we deposited two new alleles in GenBank at accession number KT869022.1 and KU959596.1.

For *IFN-γ* receptor, only eight microsatellites were recorded in present study (table (5) and figure (2)). In another study was done by Ding *et al*., (2015) found that there were thirteen extra microsatellites in population of China [13]. So further than microsatellites which mentioned in table (5), $(CA)_{18}$, $(CA)_{23}$, $(CA)_{24}$, $(CA)_{25}$ and $(CA)_{26}$. The alignment of CA motifs associated with TB infection in figure (2). CA_{17} and CA_{22} the most dominant motifs in-patient that absent with controls while CA_{14} present only with controls.

The mean IFN-g serum level (Figure (3) was significantly depressed in Patients with active TB (26.92 pg. /ml) compared with healthy control (28.40 pg./ ml), with standard deviation (5.29) and (10.73) respectively.

DISCUSSION

The number of SNPs is dfference from population to other and also between various ethnics [14]. Pires Lopes, *et al*. had studied target fragment of IFN-γ promotor region in the Brazilian. He had found seven (SNPs) [12].

Table 1: Personal data of patients

Age group						
5-14	**15-24**	**25-34**	**35-44**	**45-54**	**54-64**	**65>**
6	16	10	11	9	12	10
Gender Distribution		**Case notifications**				
Male	**Female**	**New positive**	**Relapse**	**Default**	**follow up**	**MDR(chronic)**
33	41	39	14	1	9	11
Geographic distribution						
Al Hartha	**Zubir**	**Shut AlArab**	**Center of province**	**Abu-AlKhassib**	**Al-Qurna**	**Al Mudaina**
6	**14**	**4**	**24**	**9**	**11**	**6**

Table 2: Primers using in PCR amplification of INF-γ and INFR1 gene

Primers	Sequence	Optimize TA	product size	Reference
IFN-F	5'-GGAACTCCCCCTGGGAATATTCT-3'	65.3℃	863 bp	[12]
INF-R	5'- AGCTGATCAGGTCCAAAGGA -3'			
INFR1-F	5'- TTCCTCGAAATATACTGCATCA -3'	56 ℃	190 bp	[13]
INFR1-R	5'- TATTGTAACTCATGCTGATGAT -3'			

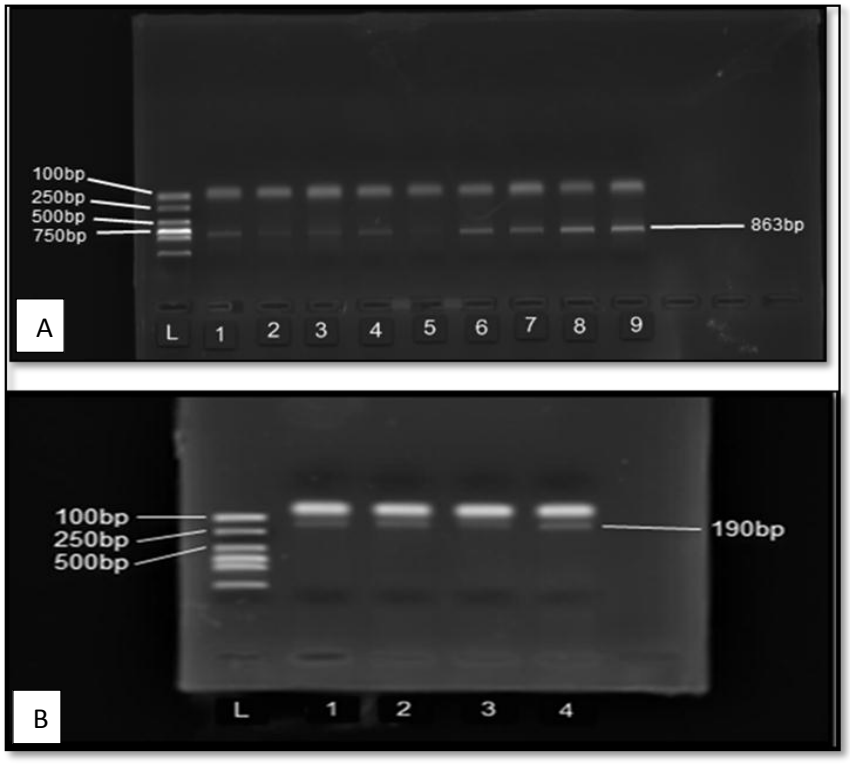

Fig 1: (A).PCR products of the DNA amplicons of IFN-γ visualized by 1% agarose gel electrophoresis, for 1 hour in (50 V), product sizes were determined by comparison with 2000bp marker. Lane L: 2000bp DNA marker, lanes 1-9: IFN-γ bands.(B) PCR products of the DNA amplicons of IFN-γ receptor visualized by 2% agarose gel electrophoresis, for 1 hour in (50 V), product sizes were determined by comparison with 2000bp marker. Lane L: 2000bp DNA marker, lanes 1-4: IFN-γ receptor bands.

Table 3: Five main SNP types, their frequency according to the positions on IFN-γ promotor and percentage of each SNP from total population SNPs

SNPs	G→C	G→A	G→T	A→T	C→A	A→G
SNPs position Frequency	0.2	0.2	0.2	0.1	0.1	0.1
Percentage(n=42)	19%	19%	33.5%	9.5%	9.5%	9.5%

Table 4: Genotype and allele frequencies of SNPs within IFNG promotor in Basra province. The expected genotype frequencies were consistent with Hardly Weinberg Equilibrium (HWE).

Locus INF-γ	Genotype	Patients (n=74)	Healthy control (n=74)	Absolute Frequency	Allele frequency	p-value
				P.	P.	P.
-768	GG CG f (C)	70 4 4	GG	0.9467 0.0527	0.0007	0.811
-713	GG AG f (A)	70 4 4	GG	0.9467 0.0527	0.0007	0.811
-714	GG TG F (T)	64 10 10	GG	0.8694 0.126	0.004	0.533
-700	GG CG f (C)	70 4 4	GG	0.9467 0.0527	0.0007	0.811
-563	AA TA f(T)	70 4 4	AA	0.9467 0.0527	0.0007	0.811
-149	GG AG f(A)	70 4 4	GG	0.9467 0.0527	0.0007	0.811
-146	CC AC f(A)	70 4 4	CC	0.9467 0.0527	0.0007	0.811
-145	AA GA f(A)	70 4 4	AA	0.9467 0.0527	0.0007	0.811
-62	GG TG f(T)	70 4 4	GG	0.9467 0.0527	0.0007	0.811

Table 5: Frequency of (CA) n alleles in the INFGR1 gene in the TB patients and controls.

Allele	*Patients (%)*	*Controls (%)*	*t-statistic*	*p-value*	*PIC*
(CA)12	*31(42%)*	*29(39.2%)*	*0.372*	*0.7106*	*0.49*
(CA)13	*8(10.8)*	*20(27.1%)*	*2.530*	*0.0125*	*0.99*
(CA)14	*0*	*5(6.7%)*	*2.265*	*0.0250*	*0.99*
(CA)17	*6(8.1%)*	*0*	*2.499*	*0.0135*	*0.99*
(CA)19	*6(8.1%)*	*10(13.6%)*	*1.076*	*0.2838*	*0.91*
(CA)20	*6(8.1%)*	*5(6.7%)*	*0.325*	*0.7454*	*0.44*
(CA)21	*6(8.1%)*	*5(6.7%)*	*0.325*	*0.7454*	*0.44*
(CA)22	*11(14.8%)*	*0*	*3.439*	*0.0008*	*0.99*

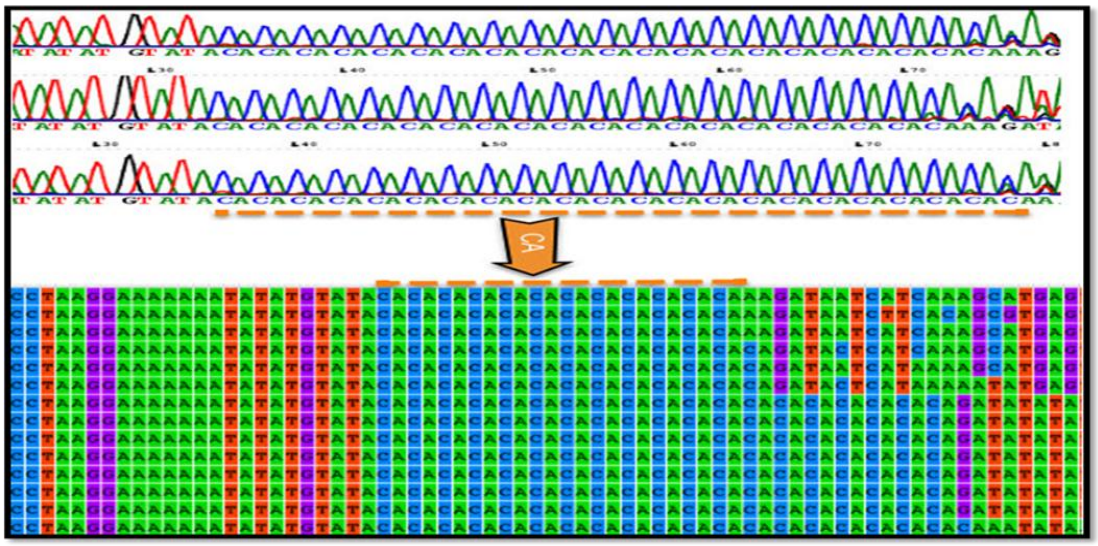

Figure 2: The alignment of CA motifs associated with TB infection, which appeared highly frequency in (CA) repeats, chromatograms alignment, are constructed by DNAdynano software and MEGA-6 software.

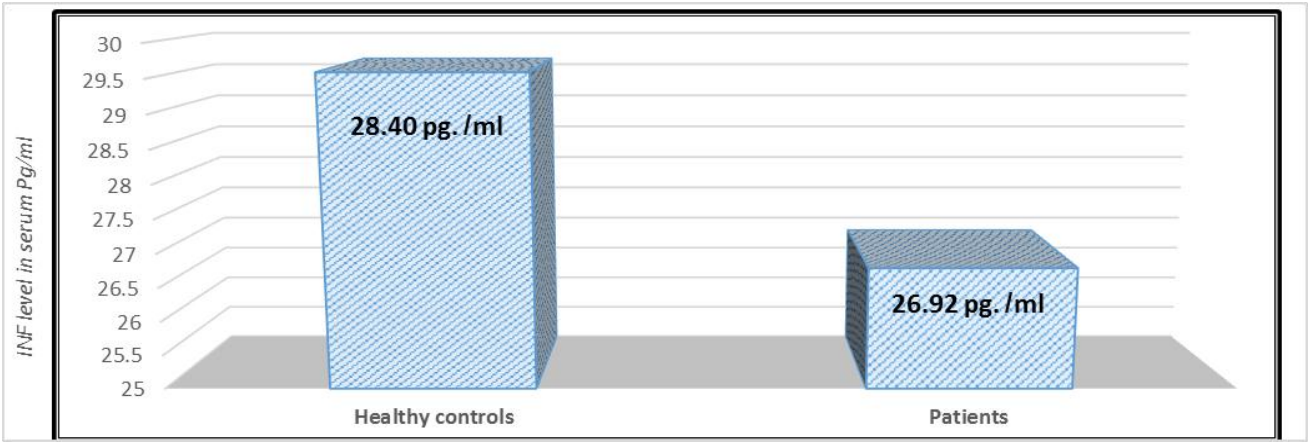

Figure 3: IFN-γ level in patients and healthy controls. The mean IFN-g serum level was significantly depressed in Patients with active TB compared with healthy control

Exactly, no previous genetic study had been done for population in Basra province. In further study, we studied extensively, the reality of tuberculosis disease in the province of Basra and reached unequivocally fact that there are genetic factors beyond re-emergent of TB in Basra society [15].

The immunopathogenesis of TB explained several genes coding for different cytokines, which have important roles in the immunopathogenesis of TB, they might affect a host's susceptibility to infection with TB. [15]. Although cytokine genes have a low frequency of genetic polymorphisms, but recently, several studies clearly demonstrated that mutations located on promotor regions or coding regions of the genes, as this defect represents as host factors impacting susceptibility to TB [17]. INF-γ gene is one of many non-HLA genes that implicate in tuberculosis susceptibility [6].

However, the conflicting data reported may be assigned to a number of various factors, like genetic background, types of studies; ethnicity and clinical status of patients with tuberculosis may be related with a definite genetic profile [18]. In present study, the results showed that there were nine single nucleotide polymorphisms (SNPs), found in IFN-γ promotor of patients but not control (table (4)), the number of SNPs is different from population to another and also between various ethnics [14]. Target fragment of IFN-γ promotor region had been studied in the Brazilian by Pires Lopes, *et al.* and he had found seven (SNPs) [12].

IFN-γ promotor contains a highly conserved region from positions -117 to -47 furthermore contains two sub-regions that can be complexes with proteins, in this region we recorded 8 SNPs. The sub-proximal region (-90 to -65) shows strong homology to the IL-2 promotor [19]. Transcription factors activate transcription of *IFN-γ* by binding to promotor region. In the other hand, inhibiting factors link in other regions influencing transcription [20, 14]. Due to the role for *IFN-γ* gene in activating the immune response, there is widely interesting in investigating the polymorphic sites within this cytokine promotor.

Concerning the fifth intron of human IFNGR1, many studies referred to a microsatellite polymorphism found. While studies also reported the relationship between IFNGR1 variants and

risk of TB [21, 22]. Interferon gamma receptor1 variant is susceptible to infection. Suggested that the gene of INFGR1 may have some effects on the development of tuberculosis infection with specific CA repeat or repeats. Results exhibited that the alleles CA_{12}, CA_{20} and CA_{21} appear to be present on infected and healthy individuals.

According to *t-value,* there were no significant differences between the two infected and healthy groups. In addition, PIC value was less than (0.5) this indicated that there is no association between $(CA)_{12}$ repeat and susceptibility to infection with (TB), polymorphic information content (PIC) was calculated according to Botstein *et al*., (table (3) [22]. If PIC value is greater than 0.5, the marker locus is highly informative but if PIC value is from 0.25 to 0.5, the marker is reasonably informative and if PIC is less than 0.25, the marker is only slightly informative. The results are coincided with what Sahiratmadja *et al*., found in Indonesia [23].

The individuals which had $(CA)_{17}$ and $(CA)_{22}$ appeared to be the most susceptible to infection with (TB). The t-value was showed that there was significant differences between the patients and controls, with PIC larger than (0.5), therefore it was indicated that these two variable number of tandem repeats are highly correlated with patients susceptibility to infection. Interferon-*γ* (IFN-*γ*) and INFGR1 present in neighboring locus. The $(CA)_{22}$ allele was correlated in transcription of IFN-γ. SNPs might affect the transcription of the *IFN-γ* gene, which may make the people whose have $(CA)_{22}$ repeat too hit with the disease [24]. The $(CA)_{13}$, $(CA)_{14}$ and $(CA)_{19}$ repeats of *IFNGR1* is responsible for protection against tuberculosis, The t-value was showed that there were significant differences between the patients and controls groups, with PIC larger than (0.5). The results were highly constant with Sahiratmadja *et al*., when he studied Indonesian population, also the results mismatch what Ding *et al*., was found in his study of Chinese population [23, 13].

The decrease in IFN-γ level in the serum of patients was accurately showed that polymorphism in this region can pose as a risk factor increase the susceptibility to TB. Th1/Th2 cytokine balance plays a key role in controlling TB infection, the major immunologic host defense mechanism for TB is based on a Th1-type cytokine response, including IFN-γ [25, 26]. Polymorphisms in promotor region, related to cytokine production. Such as this alteration might be lower the ability of transcription factor to combine with proper site of regulatory region (promotor), which may influence negatively on secretion of these cytokines [27].

Individuals carrying low IFN-γ-producing genotypes a higher risk of developing active TB [6]. Many further studies revealed elevation in IFN-γ level but without any combination with the type of polymorphism in IFN-γ gene [28, 29]. Table (5) combines among *IFN-γ* promotor, *IFN-γ* receptor and *IFN-γ* level in serum, so there was a decrease in IFN-γ level during the infection with TB; this reduction may be become as risk factor added to the other risk factors. Hashemi *et al*. when he studied the population of Zahedan, Southeast Iran, he had found that there is association between single nucleotide polymorphism of IFN-γ +874T/A and pulmonary tuberculosis (PTB) in case-control study [30]. Hashem's study and many other studies like those that it was performed in ethnically homogeneous populations, therefore, many of the associations described for a particular allelic variant in a certain gene may not represent genetic risk factor in other populations.

CONCLUSION

Tuberculosis remains a threat to all sectors of societies in all developed and developing countries of the world. In province of Basra as part of Iraq in spite of strict treatment and vaccination programs, the number of new infections with TB sound to be increasing instead of decreasing and vanishing. Tuberculosis affects both sexes, in addition, that all of age groups of population are targeted by infection with tuberculosis. The idea which refers to that there is a genetic defect in certain genes, the most suitable idea the explains re-outbreaks from time to time.

IFN-γ is very important in the immune response and that mutations that interfere with their production may influence the outcome of active tuberculosis. The single nucleotide polymorphisms (SNPs) and the difference in microsatellites that found in *IFN-γ* promotor and *IFN-γ* receptor respectively of patients but not control may lead to lessening IFN-g level in serum.

RECOMMENDATION

Although it is clear that, there are genetic defects in some genes generally in Iraqi population and especially in Basra province. There is no reference data upon which we based to evaluate which direction to turn to this genetic variation. Therefore, we strongly recommend study the genetic factors that lead to increasing the susceptibility to tuberculosis disease in the Iraqi community.

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