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Original Research Article

Molecular Study of two Types of Mutations in Promoters of IL-2 and IL-10 Genes in Iraqi Patients with Tuberculosis

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Tuberculosis remains as a black page in the ancient and modern history book of humanity. *Mycobacterium tuberculosis* is the main causative agent of TB in human. A defect in the genes of the immune response is the most plausible explanation for susceptibility of some people, and resistance of others to TB. Cytokines play a critical role in interactions and integration between the cells of immune system, which leads to effective defense against TB, among cytokines IL-2 and IL-10, which have regulatory role in immune response. The main goal of this study is to produce molecular analysis for promoters of IL-2 and IL-10 genes in Iraqi patients with TB. Seventy-four blood specimens were collected from 74 patients in the Institute of the Tuberculosis and Chest Disease–Basra city, blood specimens also collected from 74 healthy individuals as control. Extracted DNA was amplified using two sets of specific primers for promoter regions of IL-2 and IL-10 genes; purified amplicons were sequenced and were analyzed using specific software. Genotyping of IL-10 promoter displayed that range from 19 to 25 motifs but without 23-motif repeat. Individuals with CA21, CA22 and CA24 appeared to be the most susceptible to infection with (TB) with PIC larger than (0.5). CA20 is responsible for protective against tuberculosis, the t-value showed that there was significant differences between the patients and control groups, with PIC larger than (0.5). The results of the present study showed that there are two SNPs recorded in the amplified region of promoter of IL-2, which are A→C and T→G with frequencies of 0.1 and 0.19 respectively.

Keywords: Interleukin-2, Interleukin-10, *Mycobacterium tuberculosis*.

INTRODUCTION

Tuberculosis (TB) is one of the infectious diseases, which is spread from one individual to another by air droplet through cough, sneezing and song. A cough, weight loss, hungriness, fever, drenching night perspiring and extreme tiredness or lack of energy are the main common symptoms of this disease. *Mycobacterium tuberculosis* (MTB) is the main causative agent of TB (Brites and Gagneux, 2015); Statistics indicate that one-third of the world's population is harboring a latent TB infection, approximately 1.5 million deaths occurring annually from the active disease coming in second after human immunodeficiency virus (HIV) as a cause of death worldwide.

Familial clustering data, animal models, twin studies and complex segregation analysis and many other studies clearly demonstrated that there is a genetic basis to accept the incidence of tuberculosis (Pachecoa and Moraes, 2009; Hanta *et al*., 2012; Abel *et al*., 2014 and Fol *et al*., 2015**)**. Determination of the host genes, which are responsible for susceptibility and resistance to TB may lead to a better understanding of the pathogenesis of MTB also it may have a significant role in the prevention of tuberculosis which in turn could lead to development of more effective treatment and vaccines (Hu *et al*., 2015). Cytokines play a critical role in the defense against bacterial infections, both directly, by the inhibition of bacterial replication, and indirectly, through determination of the prevalent Th1/Th2 pattern of host immune response (Rynda-Apple *et al*., 2015; Yan *et al*., 2016). Interleukin-2 (IL-2) and Interleukin-10 (IL-10) are among the most important cytokines which play a great role as regulatory cytokines for host immunity against pathogens.

IL-2 gene is located on chromosome 4 and it has a crucial role in generating an immune response by inducing lymphocytes specific for an antigen, so it is called lymphokine (AL-khafaji *et al*., 2015). It is well known that IL-2 has an impact on macrophages activation and direct cytotoxicity of T-cells, which accounts for protective role of IL-2 against TB (AL-khafaji *et al*., 2015; Kobashi *et al.,* 2015). IL-10, which is

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produced by triggered macrophages, natural killer (NK) cells, dendritic cells (DCs), mast cells, B cells, and regulatory T cell subsets, is known to have macrophage-deactivating characteristics, IL-10 has critical role in bacterial infections (Kumar *et al*., 2015; Abdalla *et al*., 2016). IL-10 can reduce the production on INF- γ so the increase in IL-10 levels appears to support the mycobacterial survival in the host this means the increase in IL-10 production can suppress the immune response (Cavalcanti *et al*., 2012). IL-10 for human is coded by gene with lengths about 4.7 kb, which is found on chromosome 1q31-32 and contains five exons.

Cytokines play a critical role in interactions and integration between the cells of immune system, which leads to an effective defense against TB. The main aim of the present study is to find out the main types of mutations, which occurs in the promoter region of two genes coded for two of the most important regulatory cytokines, which are IL-2 and IL-10 and possible correlation between these mutations and outcome of TB infections.

MATERIALS AND METHODS

The specimens were collected at the institute of the tuberculosis and chest disease–of Basra province from February to September 2015. Seventy-four patients (33) male and (41) female with pulmonary TB, with average age between (38.73±23 years) were included in present study, while 74 volunteers, (36) male and (38) female, age mean between (35.19±7.86 years) were accredited in the current study as healthy control (HC). Blood samples were collected from each patient and control by vein puncture using disposable syringes. 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 IL-2 promoter and IL-10 promoter were used as shown in table (1). 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 IL-2 were initial denaturation at 95˚C for 5 min., followed by 35 cycles consisting of 30 sec. at 95°C, 45 sec. at 59°C and 35 sec. at 72°C with a final extension at 72°C for 10 min.

The PCR conditions for amplifying IL-10 promoter gene were initial denaturation at 94˚C for 5 min., followed by 35 cycles consist of 1 min. at 94°C, 1 min. at 63.3°C and 1 min. at 72°C with a final extension at 72°C for 5 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 came back from the company ABI and text file, DNA Dynamo software was used to analyse the data results, multiple alignment for high-quality sequences were done for each other plus reference sequence at GenBank performed to find DNA polymorphism within sequences.

RESULTS

Promoters of IL-**2** Gene and IL-10 were successfully amplified. The amplicons of the each promoter were visualized by agarose gel electrophoresis, whilst PCR product sizes were determined by comparison with marker as in figure (1-A, B).
Two SNPs were recorded in the amplified region of promoter of IL-2, A→C and T→G at positions -312 and -330 respectively (table 2).

The results of sequencing revealed that there are varying types of microsatellite in promoter of IL-10 (table 3), from which CA motif repeats were the most dominant microsatellite. A range from 19 to 25 motifs but without 23-motif repeat. Individuals with CA_{21}, CA_{22} and CA_{24} appeared to be the most susceptible to infection with (TB). The t-value showed that there was significant differences between the patients and controls groups, with PIC larger than (0.5).

CA_{20} is responsible for protection against tuberculosis, the t-value showed that there were significant differences between the patients and controls groups, with PIC larger than (0.5). CA_{19} and CA_{25} seem to be present on infected and healthy individuals, according to t-value, no significant differences existed between the two infected and healthy groups. In addition, PIC value was less than (0.5) this indicated that there is no association between these repeats and susceptibility to infection with (TB).

DISCUSSION

The number of SNPs differs from one population to another and also between various ethnics (Lee et al., 2015). IL-2 plays an essential role in generating immune response and it can affect the course of mycobacterial infections, either alone or in association with other cytokines. T→G mutation is well known in literature and is referred to as (rs2069762), while A→C SNPs at position -312 not previously mentioned (Butov, 2015).

T→G SNPs in IL-2 promoter has been found to have different effect on IL-2 gene expression, in one population it increases the expression of this cytokine like Chinese population (Hu *et al*., 2015). While for T→G SNPs, no significant association was shown in the expression of IL-2 in other population like Macedonian population by Trajkov *et a*l., (2009). Sivangala *et al*., (2013) found that T→G decreased the IL-2 level in Caucasian population which made that group more susceptible than others did to infection with TB.

For IL-10, the present study focused on microsatellite with CA motif repeat because firstly, this repeats occurred in the middle of sequences of target samples, by which this repeats were free of sequencing errors. Secondly, CA repeats only repeats that varied in length among patients but not in controls. Finally, some literature mentioned that this repeats might present in promoter regions of some promoters of cytokines genes, such as CA repeats in first and fourth intron of INF-γ (Wu et al., 2015).

IL-10 role is as a general inhibitor of proliferative and cytokine responses of both T helper (Th1) and Th2 cells (Spinasse *et al*., 2012). IL-10 does an anti-inflammatory action by restraining the production of cytokines such as IL-1α, IL-1β, IL-6, IL-8, IL-12, and tumor necrosis factor-alpha in activated macrophage and interferon gamma in T cells (Ke *et al*., 2015). The IL-10 promoter is extremely polymorphic region, primarily IL-10 has three single nucleotide polymorphisms (SNPs) present at positions -1082, -819, and -592.

Table 1: Primers using in PCR amplification of IL-2 and IL-10 gene

Primers		Sequence	product size	Reference
IL-2	sense	5′-AAGAGTCATCAGAAGAGGAA-3′	150 bp	Sivangala *et al.*,2013
IL-2	antsense	5′- AGCTGATCAGGTCCAAAGGA-3′		
IL-10	sense	5′-TTCCCCAGGTAGAGCAACAC-3′	565bp	Spinasse *et al.*,2012
IL-10	atisense	5′-GGCACATGTTTCCACCTCTT-3′		

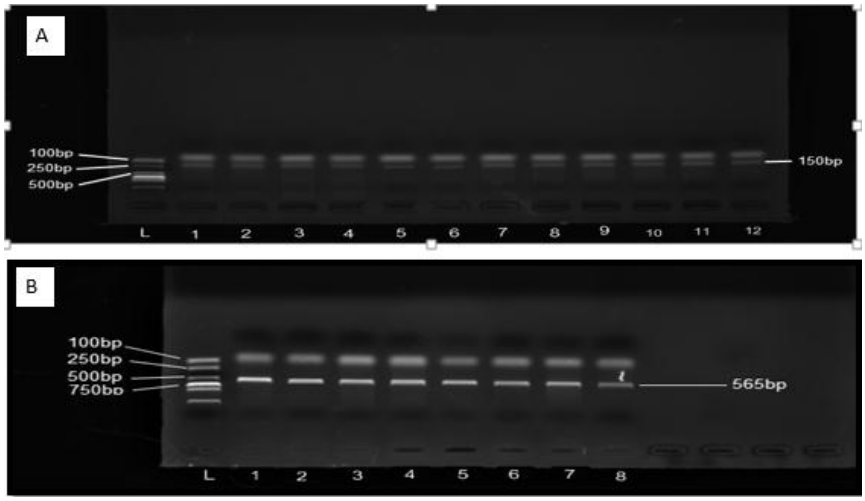

Figure 1: (A).PCR products of the DNA amplicons of IL-2 visualized by 1% agarose gel electrophoresis, for 1 hour in (50 V), product sizes were determined by comparison with 2000bp marker. Lane L: 2000bp DNA marker, lanes 1-12: IL-2 bands.(B) PCR products of the DNA amplicons of IL-10 visualized by 2% agarose gel electrophoresis, for 1 hour in (50 V), product sizes were determined by comparison with 2000bp marker. Lane L: 2000bp DNA marker, lanes 1-8: IL-10 bands**.**

Table 2: The genotype and allele frequencies of the identified IL-2 SNPs in the completely studied population (148samples)

Locus IL-2	Genotype	Patients (n=74)	Healthy control (n=74)	Absolute Frequency	Allele frequency	p-value
				P.	P.	P.
-312	*AA* *CA* *f(C)*	*66* *8* *8*	*74* *0* *0*	*0.89* *0.1*	*0.002*	*0.623*
-330	*TT* *GT* *f(G)*	*58* *16* *16*	*74* *0* *0*	*0.79* *0.19*	*0.01*	*0.2971*

Table 3: Frequency of $(CA)_n$ alleles in the I-10 gene in the TB patients and controls

Allele	*Patients (%)*	*Controls (%)*	*t-statistic*	*p-value*	*PIC*
(CA)25	*13(17.5%)*	*12(16.2%)*	*0.211*	*0.8330*	*0.3*
(CA)24	*25(33.9)*	*12(16.2%)*	*2.485*	*0.0141*	*0.99*
(CA)22	*14(18.9%)*	*5(6.7%)*	*2.221*	*0.0279*	*0.99*
(CA)21	*17(22.9%)*	*6(8.1%)*	*2.488*	*0.0140*	*0.99*
(CA)20	*3(4%)*	*18(24.3%)*	*2.543*	*0.005*	*0.99*
(CA)19	*2(2.8%)*	*21(28.5%)*	*4.303*	*0.000*	*1.00*

Studies have shown that these SNPs have been found to correlate with the production of IL-10 (Gao et al., 2015), GCC haplotype of peripheral blood mononuclear cells was related to abundant IL-10 production, whereas the ATA haplotype was correlated with low levels of IL-10 production. The amplicon region in the present study included position -592, but did not included -1082 and -819 positions. So for CA repeats > 20, C present in position -592, therefore may be high expression especially with former G and later C, which leads to increase individuals' chance to infect with TB by inhibiting activation of T-cells and the myeloid cells, including monocytes, dendritic cells and macrophages. When CA>20, C is not present in position -592 leading to lower expression for IL-10. These results agree with what Ke *et al*., 2015 mentioned that -592A/C polymorphism is dominant among Asians.

CONCLUSION

There are more than one type of mutations pushing toward increasing susceptibility of some individuals to TB, mutation in promoter region of regulatory cytokines such as IL-2 and IL-10 plays critical role in altering immune response for the sake of pathogen.

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