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Analysis of *CTLA-4* (+49A/G) Gene Polymorphism and the Risk of Pulmonary Tuberculosis in Babylon province of Iraq

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Abstract

Background: Pulmonary tuberculosis (pTB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*). It typically affects the lungs, but can also affect other sites (extra- pulmonary TB). The disease is spread when sick individuals expel bacteria into the air, for example by coughing.

Aim of Study: To highlight the effect of *CTLA-4* (+49A/G) gene polymorphism on the risk of pulmonary Tuberculosis (pTB).

Patients and Methods: This case-controlled study used single specific primer-polymerase chain reaction (SSP-PCR) to analyze the *CTLA-4* (+49A/G) gene polymorphism in 60 patients with pTB who were referred to consultant clinic for respiratory diseases in Hilla – Babylon province/ Iraq during the period from December 2017 to July 2018, and 60 healthy persons' control. Blood samples were collected from both groups according to the standard methods.

Results: Data analysis revealed that the frequencies of *AA*, *AG* and *GG* genotypes in patients were 73.33%, 23.33%, and 3.34% respectively. In controls, this frequency was 51.67%, 35%, and 13.33% respectively. Logistic regression test detected a significant difference in the frequency of the (*GG* genotype) mutant homozygous of this polymorphism between patients and controls (3.34% versus 13.33%), The *GG* genotype of *CTLA*-4(+49A/G) showed a significantly decreased risk of pulmonary tuberculosis disease (OR= 0.18, 95% CI= 0.04 – 0.88, *P* value = 0.035).

Conclusion: The *GG* genotype of *CTLA-4* may decrease the risk of pTB.

Keywords: *Pulmonary Tuberculosis*; *Mycobacterium*; *CTLA-4*.

Introduction

Tuberculosis (TB) has existed for millennia and remains a major global health problem. It causes ill-health for approximately 10 million people each year and is one of the top ten causes of death worldwide. It has been the leading cause of death from a single infectious agent[(1)]. The major risk factors that increase early death of TB patients are being positive for human immunodeficiency virus (HIV), being of old age, being underweight or undergoing retreatment[(2)]. About one-third of the world's population are estimated to be infected with *M. tuberculosis*, albeit mostly without clinical symptoms. These silent carriers bear a life time risk of developing active disease, with more than 95% of cases and deaths occurring in the developing world[(3)]. Population genetic studies have made significant contributions to reveal the role of human genetic variation in susceptibility to TB infection[(4)]. Macrophages and neutrophils play a decisive role in host responses to intracellular bacteria including the agent of tuberculosis, the role of both reactive nitrogen intermediate (RNI) and reactive oxygen intermediate (ROI) as central mediators of innate immune defense in human *M. tuberculosis* infection is well established[(5)].

The entry of *M. tuberculosis* into the body induces activation of cellular immune mechanisms which play important roles in the mechanism of T cells. TH1 cells induce macrophage activation and phagocytosis reactions. Eradication of these pathogens requires secondary administrative cellular immune mechanisms such as cytotoxic T lymphocytes (CTL)[(6)].

CTL-associated antigen 4 (*CTLA-4*) is a CD28 receptor that inhibits T cell proliferation through combination with B7 molecules. The human *CTLA-4* gene is located on chromosome 2q33. The *CTLA-4* (rs231775) polymorphism is located in the first exon of *CTLA-4* +49A/G base substitution can cause a change from threonine to alanine amino acid in the coding region of *CTLA-4* [7]. [8], revealed a significant relationship between the *GG* genotype of CTLA-4 and the increased risk of TB.

The present study aimed to investigate a possible correlation between *CTLA-4*+49A/G gene polymorphism and the risk of pTB in a sample of the Iraqi population.

Materials and Method

Study Groups:

This study designed into two groups. The first group involved 60 pTB patients. Those patients were referred to consultant clinic for respiratory diseases in Hilla – Iraq during the period from December 2017 to July 2018. All patients were subjected to full clinical and radiological examinations prior to laboratory examination. The subjects with pTB were selected from those who had a confirmed diagnosis by a healthcare professional and who presented clinical symptoms, radiological evidence and positive sputum acid-fast bacillus (AFB) smears. The second group involved 60 apparently healthy individuals were considered as control group. This control subjects had no previous history of pTB, autoimmune disease, diabetes, and chronic disease.

Collection of Blood Samples

The blood was collected by venous procedure. The site of venipuncture is sterilized by 2% iodine. Five milliliters of venous blood were collected from each participant and kept in EDTA tube. A total of 120 blood samples were collected (60 subjects with pTB patients and 60 healthy controls). DNA extraction was accomplished using (Favorgen/ Taiwan) kit according to the manufacturer's instructions. After estimation of the quality of DNA by Nanodrop, the Specific Sequence Primer (SSP-PCR) method was used to analyze the genotyping of *CTLA4* (+49A/G) with the primers given in (Table -2). The PCR conditions for the amplification of *CTLA-4* were as follows: initial denaturation phase comprised 5 minutes at 94°C; next 30 cycles (1 minutes at 94°C, 1 minutes at 57°C, and 1 minutes at 72°C); then, seven minutes at 72°C. The samples were stored at 4°C until electrophoresis as in (figure -1).

Statistical Analysis

The Statistical Package for the Social sciences (SPSS, version 20) was used for statistical analysis. Risk association between the genotype and TB susceptibility was estimated by the calculation of adjusted odd ratio and 95% confidence intervals using multivariate logistic regression. For this analysis, subjects who were homozygous for the wild type genotype were considered as reference, and polymorphisms as dependent variables. Chi- square was used for testing the deviation from Hardy- Weinberg equilibrium, and to compare between patients and control. A *P*-value < 0.05 was considered statistically significant.

Results

The demographic characteristics of study population for pTB patients and controls groups were involved the following factors as in (Table -1).

Table- 1: Demographics characteristic of study population

Variable	**Patients group No (%)**	**Control group No (%)**	***P*- value**
- Age groups 10-30 31-50 51-70	 29 (48%) 18 (31%) 13 (21%)	 23 (38%) 22 (36%) 15 (26%)	 0.269 0.439 0.666

Cont... **Table- 1: Demographics characteristic of study population**

- Gender Male Female	 35 (58%) 25 (42%)	 41 (68%) 19 (32%)	0.256
- Residence Rural Urban	 42 (70%) 18 (30%)	 25 (42%) 35 (58%)	0.002
- Smoking Smoker Non-smoker	 24 (40%) 36 (60%)	 20 (30%) 40 (70%)	0.444
- Vaccination Vaccinated Non vaccinated	 33 (55%) 27 (45%)	 43 (72%) 17 (28%)	0.058
- BMI <25 kg/m^2 ≥25 kg/m^2	44 (73.33%) 16 (26.67%)	16 (26.67%) 44 (73.33%)	**0.001**

The *AA* genotype was found in 44 (73.33%) subjects with pTB as well as in 31 (51.67%) controls subjects. The *AG* genotype was observed in 14 (23.33%) subjects with pTB and 21 (35%) control subjects. The *GG* genotype was observed in 2 (3.34%) patients with pTB and 8 (13.33%) control subject. The A allele frequency was 102 (85%) in subjects with pTB and 83 (69.17%) in control subjects. Allele frequency for the G allele was 18 (15%) in subjects with pTB and 37 (30.83%) in control subjects as in (Table -3).

Table – 2: The Primers of *CTLA-4* gene and their corresponding genes used in the present study

Types of primer	**Primer sequence (5'-3')**	**Product Size(bp)**	**Reference**
Forward	GTGGGTTCAAACACATTTCAAAGCTTCAGG	AA: 120bp AG: 229bp GG: 162bp	(9)*which is developed by the obligate intracellular Mycobacterium leprae (ML*
Reverse	TCCATCTTCATGCTCCAAAAGTCTCACTC		
Alleles			
A	ACAGGAGAGTGCAGGGCCAGGTCCTAGT		
G	GCACCAGGCTCAGCTGAACCTGGATG		

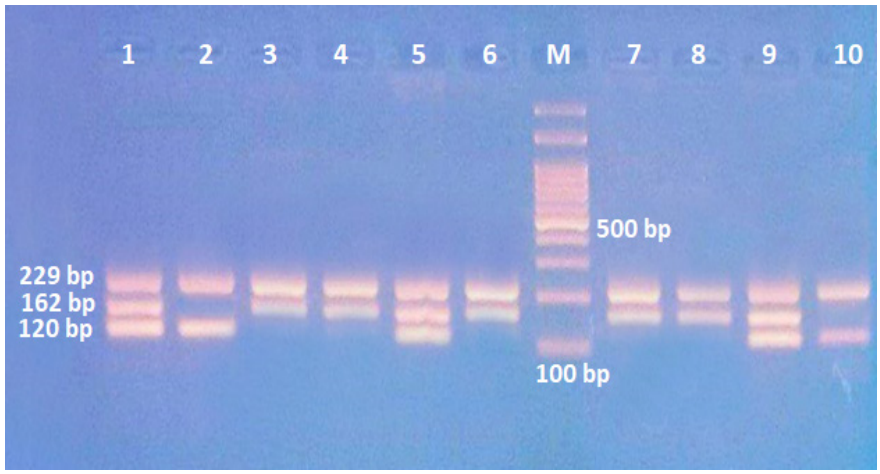
Table- 3: Genotypes and Alleles Frequency of *CTLA4* Gene +49A/G Polymorphism in Patients with pTB and the Control Group

*CTL*A-4 rs 231775	pTB Cases (60)	Controls(60)	*P*-value	OR(95%CI)
Genotypes **AA** **AG** **GG** **HWE**	 44(73.33%) 14(23.33%) 2 (3.34%) 0.51	 31(51.67%) 21(35%) 8(13.33%) 0.165	 0.036 0.07 0.035	 Reference 0.47(0.21 -1.06) 0.18(0.04-0.88)
Alleles **Allele A** **Allele G**	 102(85%) 18(15%)	 83(69.17%) 37(30.83%)	 0.005	 0.4 (0.21-0.75)

Discussion

T-cell activation is a complex process that requires >1 stimulatory signal. TCR binding to MHC provides specificity to T-cell activation, but further costimulatory signals are required. Binding of B7-1 (CD80) or B7-2 (CD86) molecules on the APC with CD28 molecules on the T cell leads to signaling within the T cell. Sufficient levels of CD28:B7-1/2 binding lead to proliferation of T cells, increased T-cell survival, and differentiation through the production of growth cytokines such as interleukin-2 (IL-2), increased energy metabolism, and upregulation of cell survival genes.

*CTL*A-4 +49 A/G binding to B7 may actually produce inhibitory signals that counteract the stimulatory signals from CD28:B7 and TCR: MHC binding[10]. Proposed mechanisms for such inhibitory signals include direct inhibition at the TCR immune synapse, inhibition of CD28 or its signaling pathway, or increased mobility of T cells leading to decreased ability to interact with APCs[11]. The polymorphism in *CTL*A-4 +49 A/G gene may reduce the risk of being infected with pTB, and can down-regulate the inhibition of cellular immune response[12]*+6230G>A (rs3087243*. The *CTL*A-4 +49 A/G (*G*) *GG* genotype are more frequent in healthy subjects than patients and may associate with a protection role against intracellular infection[13].



(Figure-1: Genotypic patterns of cytotoxic T-lymphocyte associated antigen-4 +49A/G polymorphism using SSP-PCR visualized under UV transluminator. M: DNA marker, lanes 1,5 and 9: AG genotype, lanes 3,4,6,7and 8: AA genotype, lane 2 and 10: GG genotype.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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