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SERUM LEVEL AND GENOTYPING OF CCL5 IN A SAMPLE OF IRAQI PULMONARY TUBERCULOSIS PATIENTS

Entssar S. Hafid and May K. Ismael
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
E.mail: entssarsadoon2015@gmail.com
E.mail: may.khaleel@sc.uobaghdad.edu.iq

ABSTRACT

Cysteine-cysteine chemokine ligand 5 (CCL5) is known to play an important role with immunoregulatory and inflammatory activities in the formation of granuloma during infection with *Mycobacterium tuberculosis*. About 90 subjects, involving 50 patients with pulmonary TB and 40 apparently healthy individuals (as a control group) were collected from primary health care center\AL-Sadur city sector/ Baghdad City/ Iraq, and at specialized chest and respiratory diseases center in Wassit City /Iraq during the period from January 2019 to May 2019.

The study was carried out to investigate serum level of CCL-5 of both patients and control by using enzyme linked immunosorbent assay (ELISA), and to determine the association between *CCL5* genotypes with pulmonary tuberculosis susceptibility in Iraqi population. Genotyping analysis of *CCL5 rs2107538* was performed by using amplification refractory mutation system (ARMS-PCR) method. The results revealed that serum levels of CCL-5 was significantly, ($P \leq 0.01$) increased in pulmonary tuberculosis patients compared to control. The mean ±SE of CCL-5 level in PTB patients and controls were 455.40 ±25.35 ng/L and 80.86 ± 5.96 ng/L, respectively. Analysis of H-W equilibrium revealed that CCL-5 rs2107538 GG, GA and AA genotypes in TB patient group were not in agreement with the equilibrium and there was a significant variation ($p \leq 0.05$) between the observed and expected frequencies. While control group showed an agreement with the equilibrium. At position rs2107538, *CCL-5* GG genotype showed a significant increased level of CCL-5 (531.01 ± 23.03 ng/L) in PTB patients compared to GA genotype (305.28 ± 33.45 ng/L) and AA genotype (150.27 ± 11.60 ng/L) of the patients. This study suggest that CCL-5 could be considered as a good biomarker for diagnosis of PTB, while it exclude the CCL-5 rs2107538 as major risk factor for tuberculosis in the Iraqi population.

Keywords: ARMS-PCR, *CCL5 rs2107538*, Genotyping, pulmonary tuberculosis, polymorphism.

Introduction

Tuberculosis (TB) is an airborne, infectious disease caused by *Mycobacterium tuberculosis* and commonly occurring in the lungs as pulmonary tuberculosis, but it can affect any organ in the body (Al-Dolaimi, 2018). The balance between the microorganism and the host defense systems is a determinant of progression of disease (Vyas and Goswami, 2017). A number of cytokine and chemokine signals are secreted by the host immune cells in response to MTB infection, which play a vital role against mycobacterial infections in the host immune response (Ernst, 2012; Orme *et al.,* 2015). It seems very clear that in response to MTB infection, chemokines are main regulators of immune system. CCL5 is an 8-kDa protein belongs to the CC chemokines family and known as Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES). For T-cells as well as macrophages, CCL5 is chemotactic and mediate the migration and activation of these cells into inflammatory sites (Alqumber *et al.,* 2013).

The susceptibility to contracting TB disease is likely to be multifactorial, and the active disease may develop due to several complex interactions between host and pathogen, which in turn are influenced by genetics and the environment. It is possible that several host genes are involved in this process (Bellamy, 2006; Hwang *et al.,* 2007). Therefore, this study aimed to investigate the possible association between CCL5 –403G/A (rs2107538) and PTB in a sample of Iraqi population**.**

Materials and Methods

Study groups

A total of 90 individuals were included in this study; 50 patients were diagnosed as active pulmonary tuberculosis by physicians at primary health care center\AL-Sadur city sector-Baghdad City/ Iraq, and respiratory diseases center in Wassit City/ Iraq during the period from January 2019 to May 2019. Patients were included 20 males and 30 females, with age ranged between 13 and 85 years. While 40 apparently healthy individuals (control group) were included (20 males and 20 females) with age ranged between 16 and 58 years, employed from the blood bank and Al-Zahra Teaching Hospital in Wassit City. Five ml of venous blood samples were collected from both patients and control groups, and it was divided into two tubes; 2ml of whole blood sample for DNA extraction and 3ml of whole blood dispensed in the second tube (plane tube) were serum isolated by centrifuge at 3000 rpm for 10 min. The yielded

serum were divided in to several Eppendorf tubes placed in a cool-box under aseptic condition and stored in the freezer at (-20°C) till using in further immunological test.

Assessment of CCL-5 Serum levels

Serum levels of CCL-5 from PTB patients and control groups were determined by using ELISA kit, Cat. No MBS167008 Mybiosource/ USA according to manufacture instructions at Wassit Central Public Health Laboratory-Ministry of Health-Wassit City-Iraq.

***CCL-5* rs2107538 Genotyping**

DNA samples were prepared from whole blood by using Genomic DNA

Extraction Kit (Genaid, Taiwan) following the manufacturer's instructions, and checking the concentrations and purity by using Nanodrop Software (Bioneer /korea) at 260 /280 nm. The CCL5 –403G/A (rs2107538) SNP was genotyped by using ARMS-PCR with the following primers: reverse (command): TTCTTGGGGACAACAAGGAG, forward (A allele): GGATGAGGGAAAGGCGA, forward (G allele): GGATGAGGGAAAGGCGG, Internal control-F: TGTAAACACTTGGTGCCTGATATAGCTTGA and Internal control-R CATCAGTATCTCAGCAGGTGCCACTAATCT. The primers used in this study were obtained from Macrogen, Korea. Amplification was performed under the following conditions: an initial denaturation step at 95 o C for 5 min followed by 30 cycles at 95 o C for 30s, annealing temperature for 23 s at 56◦C, and 25 s at 72◦C, with a final extension of 72◦C for 10 min. The PCR products were separated by electrophoresis in 2% agarose gels, and observed under ultraviolet light. The PCR product size was 130 bp. When the positive amplification of both PCR reactions exhibited a GA genotype, while positive amplification in only the first PCR reaction exhibited a GG genotype and finally, the positive amplification in the second reaction only showed an AA genotype of *CCL-5* rs2107538 gene (figure.1).

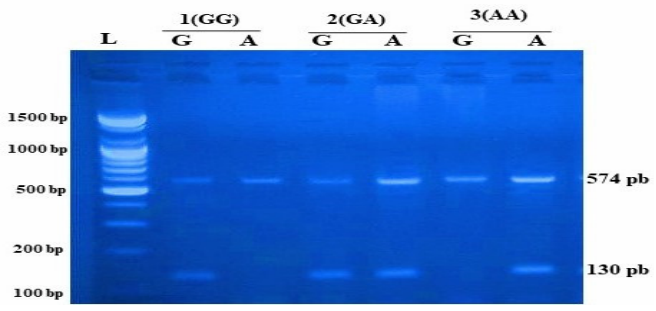

Fig. 1: Photograph of the PCR products of the CCL5 -403 G>A polymorphism using ARMS-PCR. L: DNA lader; sample 1: GA; sample2: GG; sample 3: AA.

Statistical analysis: All statistical analysis was performed using statistical analysis system (SAS) program version 9.1 for windows (SAS. Inst. Inc., Cary. N.C., USA).Results were expressed as mean ± SE. T-test, least significant difference (LSD0.05) and Chi-square test were used to analyse the results and comparisons between two groups. Differences was considered significant when P ≤ 0.05and P≤0.01. Genotypes of CCL-5 rs2107538 was presented as percentage frequencies and significant differences between their distributions in PTB patients and controls were assessed by two-tailed Fisher's exact probability (P).In addition, odds ratio (OR), etiological fraction (EF) and preventive fraction (PF) were also estimated to define the association between a genotype with the disease. These estimations were calculated by using the latest version of the WINPEPI package (including the programs and their manuals) available free online at http://www.brixtonhealth.com. Allele frequencies of genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles which is available free online athttp://www.had2know.com/academics/hardy-weinbergequilibrium-calculator-3-ale;es.html (SAS, 2012).

Results and Discussion

Serum level of CCL-5

The results of CCL-5 showed significant (*P* ≤0.01) increased in CCL-5 levels between PTB patients and controls with mean ±SE for PTB patients and controls were (455.40 ±25.35 and 82.19 ± 5.84) ng/L respectively, as shown in Figure .2

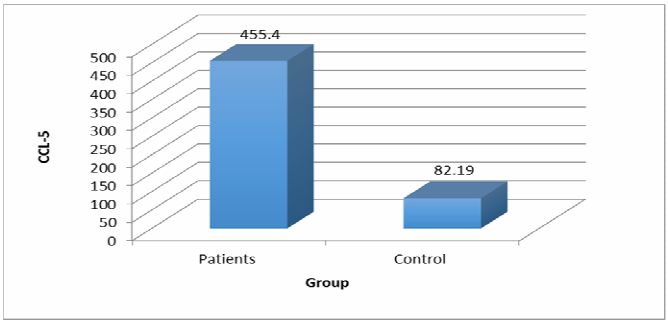

Fig. 2 : Serum level of CCL-5 in pulmonary tuberculosis patients and controls.

As a major chemokine, CCL5 known as Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) plays amajor role in co-stimulation of T cell proliferation and activation of RANTES in anti-mycobacterial immunity (Bacon *et al.,* 1995). Moreover, has a protective role in MTB infection by forming granuloma, limiting pathogen growth, and preventing lung tissue damage (Vesosky *et al.,* 2010). CCL5 (β-chemokine) are not only T-cell and macrophage chemoattractants, activating and extending T cell populations (Bacon *et al.,* 1995); but also macrophage coactivators, thus inducing a Th1 response (Dorner *et al.,* 2002).

Genotypic and allelic frequencies of the CCL-5 rs2107538 SNP in TB and control populations:

Analysis of H-W equilibrium revealed that CCL-5 rs2107538 GG, GA and AA genotypes in TB patient group were not in agreement with the equilibrium and there was a significant variation (p ≤ 0.05) between the observed and expected frequencies. While control group showed an agreement with the equilibrium as shown in table -2

Table 2: Observed and expected number with the percentage frequencies and Hardy-Weinberg (H-W) equilibrium of CCL-5 rs2107538 genotypes and alleles in TB patients and control groups.

Studied groups			CCL-5 rs2107538 genotypes or allele					HWE P<
			GG	GA	AA	G	A	
TB. patient (n:50)	Observed	NO	36	10	4	82	18	0.05
		%	72	20	8	82	18	
	Expected	NO	33.6	14.7	1.6	Not estimated		
		%	67.2	29.2	3.2			
Control (n:40)	Observed	NO	34	5	1	73	7	N.S*
		%	85	12.5	2.5	91.25	17.5	
	Expected	NO	33.3	6.3	0.3	Not estimated		
		%	83.25	9	0.75			

*Non significant

Comparison of TB patients groups to control group, the patient group showed none of the genotypes or alleles have a significant difference between patients and controls (Table. 3). The GG genotype was insignificant decreased in patients than control (72% *vs.* 85%; p> 0.05), with OR value of 0.45 and EF 0.46. While GA genotype was insignificantly increased in patients than control (20% *vs.* 12.5%; p> 0.05) with OR value of 1.75 and EF value 0.08. *G* alleles were reduced in patients than control (82% vs. 91.2%; p> 0.05) with OR value of 0.44 and EF 0.51. Alleles were increased in patients than control (18% *vs.* 17.5%; p> 0.05) with OR value of 2.29 and EF 0.08.

Table 3 : Statistical evaluations of association between *CCL-5* rs2107538 genotypes or alleles in TB patients and control groups.

CCL-5 rs2107538	Patients (n:50)		Controls (n:40)		OR	Etiological Or Preventive Fraction	Fishers Exact Probability	95% Confidence interval (C.I.)
	NO.	**%**	**NO.**	**%**				
Genotypes								
GG	**36**	**72**	**34**	**85**	**0.45**	**0.46**	**0.20**	**0.16-1.3**
GA	**10**	**20**	**5**	**12.5**	**1.75**	**0.08**	**0.4**	**0.55-5.5**
AA	**4**	**8**	**1**	**2.5**	**3.39**	**0.05**	**0.3**	**0.37-30**
Alleles								
G	**82**	**82**	**73**	**91.25**	**0.44**	**0.51**	**0.08**	**0.17-1.1**
A	**18**	**18**	**7**	**17.5**	**2.29**	**0.1**	**0.08**	**0.91-5.7**

In Moldavian patients, Varzari *et al*. (2018) found that genotypes of rs2107538 not significantly associated with TB infection .GG genotype was higher frequency (57.8%) than frequencies of GA and GG genotypes (38.1% and 4% respectively), while in control group genotype frequencies were GG (61.4%), GA (33.7%) and AA (4.9%)) . While in Sudan, Mhmoud *et al*., (2013) reported that there were no genetic associations between the rs2107538 SNPs and TB and genotypes frequencies were 43.97%, 3.66% and 52.63% for GG, GA and AA, respectively in TB patients and in control were 33.01%, 22.81% and 44.17%, respectively.

In India, Selvaraj *et al*. (2011) found that the rs2107538 genotype polymorphism not related to predisposition to contracting PTB individually. They reported that GG, GA and AA genotypes frequency were 51.4%, 38.7% and 9.9%; respectively in TB, patients while 43.1%, 46% and 10.9%, respectively in control group. Whereas, Ben-Selma *et al.,* (2011) indicated an association of the CCL5–403G/A polymorphisms with susceptibility to TB infection in Tunisian populations.

A number of infectious and inflammatory diseases have been reported to associate with variations of sequence in chemokine protein-coding and regulatory regions (Qidwai and Khan, 2016; Qidwai, 2016) thus, this study investigated whether CCL5 polymorphisms conferred susceptibility to pulmonary TB in the analyzed Iraqi patients. Our results showed that the rs2280788 genetic polymorphism is not involved in susceptibility to or protection against PTB in Iraqi patients.

Conclusion

CCL-5 could be considered as a good biomarker for diagnosis of PTB, while it excludes the CCL-5 rs2107538 as major risk factor for tuberculosis in the Iraqi population**.** However, as the present study was performed with relatively small sample size, further studies with larger sample sizes would be necessary to elucidate the role of CCL-5 polymorphisms in tuberculosis.

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