Iraqi Journal of Biotechnology, 2022, Vol. 21, No. 2, 326-330

Impact of Interleukin-21 Gene Polymorphism on IL-21 Serum Level in Iraqi Rheumatoid Arthritis Patients

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Received: 1/6/2022 Accepted: 11/9/2022 Published: December 20, 2022

Abstract: Interleukin-21 (IL-21) is a cytokine which plays a significant role in the pathogenesis and disease activity of rheumatoid arthritis (RA). Genetic polymorphisms IL-*21* gene may alter the synthesis of IL-21cytokin. To investigate the polymorphisms of the IL-21 gene promoters with RA, one single nucleotide polymorphisms (SNPs) in the promoter of the IL-21 gene (rs2221903) in 60 Iraqi patients with RA and 20 controls were evaluated using polymerase chain reaction (PCR). Also, the serum level of IL-21 was measured. No significant differences were found in the genotype distribution, allele frequency and haplotype frequency for polymorphism of IL-21 gene between the RA and controls groups. Comparison to controls, patients with RA were more likely to have high level of IL-21. The present study concluded that there was no correlation between IL-21 polymorphism (rs2221903) and RA. The level of IL-21 was highly statistically significant in RA comparing to normal individual.

Keywords: Rheumatoid Arthritis, Interleukin-21, IL-21 Polymorphism (rs2221903).

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Introduction

Rheumatoid arthritis (RA) is a systemic, inflammatory autoimmune disorder with numerous manifestations caused due to intricate chain of events (1). Cells of the leukocyte lineage such as Monocytes-macrophages, neutrophils, mastocytes and subsets of T and B cells majorly contribute to the pathogenesis of RA by secreting various cytokines and chemokines (2). The exact cause of RA is not yet known, although both genetic and environmental factors have been implicated as having a role in disease development (3**).** Interleukin 21 (IL-21), a double role cytokine discovered during the year 2000 shares similar homology with IL-2 family of cytokines (IL-2, IL-4 and IL-15), produced by T helper 17 (Th17), follicular T helper (Tfh) and natural killer T (NKT) cells (4). In recent years, IL-21 has been found to be a key player in RA pathogenesis and progression (5,6). In RA pathogenesis, IL-21 receptor (IL-21R) is highly expressed on CD4+ T cell subsets, macrophages, dendritic cells and synovial fibroblasts, these immune cell subtypes recognize the IL-21 in the microenvironment to carry out several intricate chains of events (7). Previous studies have revealed increased levels of IL-21 in RA patients, which correlated with disease activity parameters (8). It has been shown that the expression of IL-21 may be modulated by the genetic polymorphisms in genes coding IL-21(9). Genetic polymorphisms have been studied in various diseases, as factors associated with increased disease risk (10). The aim of this study to investigate the association of SNPs in

the promoter region of IL-21 (*rs2221903*) with the risk of RA in an Iraqi population.

Material and method

A group of patients representing a homogeneous sample of rheumatoid arthritis who were referred to the rheumatology unit in Baghdad Teaching Hospital was studied.

Sixty RA patients were ascertained and enrolled in the study and their age range from 25-65 years. The diagnosis was made by the consultant medical staff at the Rheumatology Unit. It was based on clinical examination, X-ray findings and laboratory tests. The diagnosed was according to the Revised diagnostic criteria established by the American College of Rheumatology (ACR), 2010, which included tender and swollen joints counts, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and rheumatoid factors (RF).

The control group was selected randomly from Iraqi population, consisted of 20 healthy individuals.

DNA extraction

Genomic DNA was extracted from venous blood samples according to the protocol ReliaPrep™ Blood gDNA Miniprep System, (Promega , USA**)** as per the manufactures instruction and stored at -20 C until use. Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of samples for downstream applications. For 1 µl of DNA, 200µl of diluted Quantifluor Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected. The DNA fragments were separated in agarose gel. A 100 ml of 1X TAE buffer was used in DNA electrophoresis, and the run was for 60 minutes at 100 volt/ 50mAmp.the agarose gel was stained with Ethidium Bromide (10mg/ml), the Ethidium bromide-stained bands in gel were visualized using Gel imaging system. Then the gel documentation system was used for document the bands

IL-21 SNP

In the current study, the single nucleotide polymorphism (SNP) was genotyped which was IL-21 (rs2221903) SNP. Analysis of data and determined sequence variation between samples of specific gene using geneious software after amplification.

Primers

The specific primers were designed for rs2221903 region of IL-21. These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl. The details of these primers which including sequence and their PCR product size are presented in table (1).

Table (1): Specific primers sequence and their PCR Product size for the IL-21 gene.

Primer Name	Sequence 5`-3`	Annealing Temp.(°C)	Product size (bp)
rs2221903-F	TGTAAAACGACGGCCAGTGCTTCCAGACA GTGCTAAAT	60	878
rs2221903-R	CAGGAAACAGCTATGACGCAGATTGCCTC TCATAAGG		

PCR components

The components of PCR reaction and conditions were shown in table (2). Also, the optimization of PCR reaction was accomplished after several trials for the precision of DNA and primers concentrations. The PCR reaction was carried out as shown in table (2).

Table (2): PCR reaction of IL-21 gene components and conditions

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	12.5
Forward primer	10	µM	1	µM	1
Reverse primer	10	µM	1	µM	1
Nuclease Free Water					8.5
DNA	10	ng/µl	2	ng/µl	2
Total volume					25
Aliquot per single rxn	23µl of Master mix per tube and add 2 µl of Template				
Steps	°C		m:		Cycle
Initial Denaturation	95		05:00		1
Denaturation	95		00:30		30
Annealing	60		00:30		
Extension	72		00:30		
Final extension	72		07:00		
Hold	10		10:00		1

Results

IL-21 SNPs in the studied groups

The SNP (rs2221903 G/A) was presented with three genotypes (GG, GA and AA) and two alleles (G and A). Analysis of Hardy-Weinberg equilibrium (HWE) in RA patients and controls revealed that the genotypes were consistent with equilibrium, and no significant differences were observed between the observed and expected genotype frequencies (Table 3).

Inspecting IL-21 genotype and allele frequencies in RA patients and controls revealed that there were no significant variation between these frequencies, although a decreased frequency of G allele (10.0 *vs.* 15.0%) and an increased frequency of A allele (90.0 *vs.* 85.0%) were observed in patients compared to controls (table 4).

Table (3): Number and percentage frequencies (observed and expected) of IL-21 gene (rs2221903SNP) Genotype and their Hardy-Weinberg equilibrium (HWE) in rheumatoid arthritis patients and controls.

Genotyping of rs2221903	Patients group frequency (%)		Control group frequency (%)	
	Observed	Expected	Observed	Expected
GG	0 (0.0)	0.60 (1.0)	0 (0.0)	0.45 (2.25)
GA	12 (20.0)	10.80 (18.0)	6 (30.0)	5.10 (25.50)
AA	48 (80.0)	48.60 (81.0)	14 (70.0)	14.45 (75.25)
Total	60 (100.0)	60 (100.0)	20 (100.0)	20 (100.0)
P-HWE	0.3894		0.4300	

Table (4): Statistical analysis of association between genotypes and alleles of *IL-21* gene (rs2221903SNP) and rheumatoid arthritis.

Genotyping of rs2221903	Patients group frequency NO (%)	Control group frequency NO (%)	OR (95% CI)	EF or PF %	Fisher's exact probability
GG	0 (0.0)	0 (0.0)	-	-	-
GA	12 (20.0)	6 (30.0)	0.58 (0.19 – 1.79)	12.5	P > 0.05
AA	48 (80.0)	14 (70.0)	1.71 (0.56 – 5.27)	33.3	P > 0.05
Total	60 (100.0)	20 (100.0)			
Alleles frequencies					
G	12 (10.0)	6 (15.0)	0.63 (0.22 – 1.79)	5.6	P > 0.05
A	108 (90.0)	34 (85.0)	1.59 (0.56 – 4.51)	33.3	

EF: Etiological fraction; PF: Preventive fraction; OR: Odds Ratio; CI: Confidence Interval.

Level of IL-21 antibodies in the sera of studied group

The studies found that IL-21 level was detected in the sera of RA patients, IL-21 level was significantly increased in RA patients (118.69 ± 13.09) compared to control group (27.89 ± 3.77) (P < 0.001), as shown in table (5).

Table (5): Distributions of studied Group according to Interleukin 21 (IL-21).

	IL-21 level mean ± SE (Unit)	
Patients group (NO)	118.69 ± 13.09	
Control group (NO)	27.89 ± 3.77	P < 0.001

Discussion

In this study we examined the genetic polymorphisms in genes coding IL-21 in patients with RA. Our results showed no significant differences in the distribution of the studied genotypes between RA patients and controls, suggesting that IL-21 SNPs are not the genetic loci predisposing to RA development. And this agrees with (11), while the serum level of IL21 in rheumatoid patients were high compared to control as shown in Table (5). And this agrees with the (12,13) in which RA patients (19.6 ± 0.79 ng/mL) displayed significantly higher levels of plasma IL-21 compared to healthy controls (2.12 ± 0.08 ng/mL) ($p < 0.0001$).

Xing *et al*, 2016. found that IL-21 can promote the proliferation of synovial tissue and pro-inflammatory cytokine production in RA patients (6) Numerous other studies have revealed the important role of IL-21 in RA activity.

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