Scientific Foundation SPIROSKI, Skopje, Republic of North Macedonia
Open Access Macedonian Journal of Medical Sciences. 2025 Mar 15; 13(1):55-60.
https://doi.org/10.3889/oamjms.2025.12003
eISSN: 1857-9655
Category: *A – Basic Sciences*
Section: *Genetics*

Assessment of Interleukin-21 Gene Polymorphisms and Protein Level in Rheumatoid Arthritis among Egyptians

Fathy M. Elfasakhany[1,2*], Mashael S. Alqahtani[1], Ahmed F. Elfasakhany[3], Ayman M. A. Elguindy[1,2,4]

[1]Department of Basic and Clinical Oral Sciences, Faculty of Dentistry, Umm Al Qura University, Saudi Arabia; [2]Department of Medical Biochemistry, Faculty of Medicine, Tanta University, Tanta, Egypt; [3]Department of Internal Medicine, Faculty of Medicine, Misr University for Science and Technology, 6 October, Giza, Egypt; [4]Department of Internal Medicine, Faculty of Medicine, Tanta University, Tanta, Egypt

Edited by: Slavica Hristomanova-Mitkovska
Citation: Elfasakhany FM, Alqahtani MS, Elfasakhany AF, Elguindy AMA. Assessment of Interleukin-21 Gene Polymorphisms and Protein Level in Rheumatoid Arthritis among Egyptians. Open Access Maced J Med Sci. 2025 Mar 15; 13(1):55-60. https://doi.org/10.3889/oamjms.2025.12003
Keywords: IL-21; rheumatoid arthritis; polymorphism; SNP; Egyptians
***Correspondence:** Fathy M. Elfasakhany. Department of Basic and Clinical Oral Sciences, Faculty of Dentistry, Umm Al Qura University, Saudi Arabia. E-mail: fmfasakhany@uqu.edu.sa
Received: 27-Nov-2024
Revised: 28-Feb-2025
Accepted: 05-Mar-2025
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Funding: This study was self-funded and did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Rheumatoid Arthritis (RA) is characterized by joint damage, persistent inflammation, and producing autoantibodies. Interleukin 21 (IL-21) and its receptor (IL 21R) have an important function in the pathogenesis of RA. High serum level of IL-21 has been related to RA and disease activity.

AIM: Here, we assessed the relationship of IL-21 polymorphisms and IL-21 serum levels with RA among Egyptians.

METHODS: DNA was separated from peripheral blood of 120 healthy controls and 120 patients with RA and genotyped for two single nucleotide polymorphisms of the IL-21 gene (rs2055979 and rs2221903) using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) technique. IL-21 serum level was assessed by ELISA.

RESULTS: The IL-21 rs2055979 AA genotype and A allele were higher in RA patients than in the control group ($p = 0.005$, OR=2.657, 95% CI=1.333-5.293 and $p = 0.019$, OR=1.932, 95% CI=1.112-3.356 respectively). The serum levels of anti-CCP, IL-21, Rheumatoid factor, C-reactive protein and ESR were higher in RA subjects in comparison to the controls ($p < 0.0001$). Furthermore, IL-21 serum level was higher in AA genotype of RA patients in comparison to the CC and CA genotypes ($p < 0.001$ and 0.041 respectively). The IL21 rs2221903 genotypes and alleles were statistically indifferent between the studied groups.

CONCLUSIONS: IL-21 rs2255979 polymorphism is associated with the susceptibility to RA in Egyptian subjects. Moreover, the elevated IL-21 levels and its association with AA genotype in RA patients indicate its importance in the pathogenesis of RA. This means that IL-21 might be used as genetic marker and would have therapeutic application in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory illness characterized by progressive joint damage and hyperplastic synovium, production of autoantibody and systemic manifestations [1]. The aetiology of RA is multifactorial and both environmental and genetic variables are suggested to have a role in the pathogenesis of RA [2].

Several immune cell types such as B and T lymphocytes, macrophages, neutrophils and monocytes are involved in the pathogenesis of RA by production of different inflammatory cytokines [3].

IL-21 is a member of class 2 cytokines and is predominantly produced by CD4 T cells and natural killer cells (NKT) [4].

IL-21 has a crucial role in B-cell activation, Th17 differentiation and antibody production so, it may have a role in the aetiology of autoimmune diseases [5]. The role of IL-21 in RA is not fully understood; however, it is detected in the synovial tissues of patients with RA [6], [7]. IL-21 performs its action by binding its receptors (IL-21R) that are expressed mainly in T and B lymphocytes, NK cells, macrophages, and dendritic cells together with some non-immune cells, like intestinal epithelial cells, fibroblasts and endothelial cells [8], [9].

Furthermore, previous studies reported the relationship of IL-21 and IL-21R polymorphisms with some autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, autoimmune thyroid disease and RA in various ethnicities [10], [11], [12], [13].

The IL-21 gene is present on chromosome 4q27 and has five exons. IL-21 rs2055979 and rs2221903 SNPs are present in the second intron. Because there is evidence that IL-21 is involved in autoimmune diseases, the presence of polymorphisms in IL-21 gene may be associated with RA susceptibility and IL-21 levels [14].

Different studies have found an association between IL-21and/or IL-21R polymorphisms and RA in various populations like Chinese [15] Netherlanders [16], Columbian [17], Algerian [18] and Mexican populations [14]. However, only few reports studied the relationship between IL-21 and/or IL-21R polymorphisms and susceptibility to RA in Egyptian population [19], [20]. The aim of the present study was to determine the relationship between IL-21 (rs2055979 and rs2221903) polymorphisms and IL-21 plasma levels and the susceptibility to RA in Egyptian population in Tanta environ, Egypt.

Materials and Methods

Subjects

This study included 120 unrelated subjects with RA and 120 controls. RA subjects were all outpatients who attended the Internal Medicine Department, Tanta University Hospital, Tanta, Egypt. Diagnosis of RA was performed as stated by the American Rheumatism Association revised data. The exclusion criteria included Diabetes mellitus, other autoimmune and neoplastic illnesses. The control subjects were randomly selected from those who either came for routine health check or at their place at work. Blood specimens were withdrawn from all subjects between 9:00 and 11:00 a.m. after fasting from 10:00-11:00 p.m. the previous day. Each specimen was divided into three thirds, one third for serum preparation, one third for ESR measurement and the last third was kept in sterile K_3EDTA (tri-potassium ethylenediaminetetraacetic acid) coated tubes for DNA separation. Samples were stored at –20 ˚C till the time of assay. Approval of this study was obtained from Ethical Committee of the school of Medicine, Tanta, Egypt and all subjects have filled written informed consents.

Clinical evaluation

Disease activity was determined using DAS28-ESR (disease activity erythrocyte sedimentation rate) score. Subjects with DAS28-ESR ≤ 2.6 were considered in remission of RA symptoms, while those with DAS28-ESR > 2.6 were considered as active RA [21], [22].

Serological and inflammatory markers assays

Rheumatoid factor was assessed using rapid latex method (Spinreact, Santa Colma, Spain) according to the manufacturer instructions. C-reactive protein (CRP) was assessed using a semiquantitative technique (Spinreact, Santa Colma, Spain) according to the manufacturer instructions. ESR was determined using Westergreen method (reported as mm/h). The Anti-cyclic citrullinated peptides (anti-CCP antibodies) were measured using commercial ELISA kit according to the manufacturer instructions (Abbexa LTD, Cambridge, UK).

IL-21 assay

IL-21 level was measured in the serum specimens using commercial human IL-21 enzyme-linked immunosorbent assay (ELISA) kit (ABCAM, Cambridge following manufacturer’s protocol. The sensitivity of the ELISA kits used in the experiment was 20 pg/ml.

DNA isolation

Isolation of DNA from venous blood was performed using DNA extraction kit as stated by the maker’s commands (Qiagen, Hilden, Germany). Aliquots of DNA were utilized for PCR amplification.

IL-21 (rs2055979 and rs2221903) genotyping

The IL-21 (rs2055979 and rs2221903) polymorphisms were assessed utilizing PCR-restriction fragment length polymorphism as described before [14]. The primers for IL-21 (rs2055979) were forward 5'- CAGCCAGGAAACTCTGGAAAGAA -3' and reverse 5'- GCTCTGAACCCAAACACTCTCATTT -3'. The primers for IL-21 (rs2221903) were forward 5'-TGGACACTGACGCCCATATTGA -3' and reverse 5'-AAGGCAGTTTAGTGGCGACAGCT-3'.

The PCR program consisted of an initial denaturation step at 94°C for 5 minutes, then 35 cycles of 94°C for 50 s, 62°C for 50 s, and a final elongation at 72°C for 5 min. The restriction enzyme NlaIII was employed to digest the PCR product of IL-21 (rs2055979) [212 bp] and the genotypes were (AA, 212 bp; CA, 212 bp, 158 bp, and 54 bp; and CC, 158 bp, 54 bp) while the MboII was used to digest IL-21 rs2221903 PCR product (230 bp) and the genotypes were (TT, 230 bp; TC, 230 bp,149 bp, and 81 bp; and CC, 149 bp, and 81 bp). The fragments were separated and visualized on 7 % agarose.

Statistical analysis

Analysis of data was performed utilizing SPSS, version 20.0. (Chicago. IL, USA). Continuous variables were analyzed utilizing Student's t test, while categorical data were assessed utilizing Chi-square (χ^2) test. One way ANOVA was used for multiple group comparison. Non-parametric data were analyzed using Kruskal–Wallis and Mann-Whitney U tests. Calculation of odds ratio (OR) was calculated with a 95% confidence interval. P value < 0.05 were considered statistically significant.

Results

Demographic, clinical and laboratory data

Demographic, clinical and laboratory data of studied groups are shown in Table 1. The study sample consisted of 240 Egyptian subjects. The controls included120 healthy subjects (87 females and 33 males) with a mean age of 48 ± 7.4 years. The RA patient group included 120 subjects (89 females and 31 males) with a mean age of 49 ± 6.7 years. The mean values of gender and age were statistically indifferent between both groups (p > 0.05). ESR, serum levels of CRP, RF, anti-CCP and IL-21 showed higher values in RA patients compared with the controls (p < 0.0001). According to DAS28-ESR score, 80.83% of patients were considered active and 19.17% were in remission.

Table 1: Demographic and laboratory data for control subjects and patients with RA

Characteristics	Control group	Patient group	*P* value
Subjects (n)	120	120	n.s.
Age (years)	48±7.4	49±6.7	n.s.
Gender (M/F)	33/87	31/89	n.s.
Disease duration (years)	-----	8.6±6.3	
ESR (mm/h)	10.25±7.52	41.35±10.65	<0.0001
CRP (mg/L)	3.43±1.56	20.23±11.46	<0.0001
RF (IU/ml)	3.73±2.16	51.98±23.94	<0.0001
Anti-CCP (U/ml)	1.58±0.61	198.62±87.4	<0.0001
IL-21 (pg/ml)	43.69±16.59	259.4±99.71	<0.0001
DAS28-ESR <2.6 (inactive)	--	23(19.17%)	
DAS28-ESR >2.6 (active)	--	97(80.83%)	

Data are shown as mean ±SD. RA: Rheumatoid arthritis; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; Anti-CCP: anti-cyclic citrullinated peptide antibodies; DAS28-ESR: Disease Activity Score 28- erythrocyte sedimentation rate. n.s.: non-significant.

Genotype and allele distributions of IL-21 (rs2055979 and rs2221903)

The distribution of genotypes and alleles of studied SNPs in both groups are shown in Table 2. The genotype distributions of IL-21 (rs2055979 and rs2221903) polymorphisms for both RA and control groups were in Hardy-Weinberg equilibrium. For IL-21 (rs2055979) polymorphism, the CC, CA and AA genotypes were 39.16 %, 41.66 % and 19.17 % respectively in the controls and were 25 %, 42.5 % and 32.5 % in RA group respectively. The distribution of C and A alleles were 60% and 40% in the controls and 46.25% and 53.75% in the RA subjects respectively.

Table 2: Genotype distribution and allele frequencies of IL-21 polymorphisms in subjects with RA and control groups

IL-21 polymorphism	Control (n =120)		RA (n =120)		$^{a}\chi^2$	*P* value	Odds ratio	95 % CI
	No	%	No	%				
rs2055979 genotypes:								
CC	47	39.16	30	25			1	
CA	50	41.66	51	42.5	2.344	0.126	1.598	0.876-2.916
AA	23	19.17	39	32.5	7.875	0.005	2.657	1.333-5.293
Alleles:								
C	144	60.00	111	46.25			1	
A	96	40.00	129	53.75	5.526	0.019	1.932	1.112-3.356
IL-21 rs2221903 genotypes:								
CC	11	9.16	4	3.34			1	
TC	50	41.67	52	43.33	3.098	0.078	2.860	0.854-9.576
TT	59	49.17	64	53.33	3.442	0.064	2.983	0.90-9.882
Alleles:								
C	72	30	60	25			1	
T	168	70	180	75	3.484	0.062	2.927	0.905-9.466

The AA genotype and A allele were higher in RA patients compared with the controls (p =0.005, OR=2.657, 95% CI=1.333-5.293 and p =0.019, OR=1.932, 95% CI=1.112-3.356 respectively). For IL-21 (rs2221903) variants, the CC, TC and TT genotypes were 9.16 %, 41.67 % and 49.17 % respectively in the controls and 3.34 %, 43.33 % and 53.33 % in subjects with RA respectively. The frequencies of the C and T alleles were 30 % and 70% in the controls and were 25% and 75% in the RA subjects respectively. There were no statistical differences between genotype and allele distribution between the studied groups.

Serum levels of IL-21 and autoantibodies in different genotypes of IL-21 (rs2055979)

The serum levels of RF, anti-CCP and IL-21 were compared between different genotypes of IL-21 (rs2055979). The serum IL-21, Anti-CCP and RF showed significant results on comparing the three genotypes (p= <0.0001, <0.0001 and 0.005 respectively) (Table 3). Two group comparisons were performed between every two genotypes for serum IL-21, Anti-CCP, RF and DAS28-ESR (Table 4).

Table 3: Comparison between different genotypes of IL-21 rs2055979 with respect to serum levels of Anti-CCP, RF and IL-21 in subjects with RA

Genotype	n	IL-21	*F	*P*	Anti-CCP	*F	*P*	RF	*F	*P*
CC	30	203.7±86.73	14.38	<0.0001	172.73±79.16	14.53	<0.0001	40.84±27.00	5.451	0.005
CA	51	248.8±93.37			179.78±72.61			53.01±22.48		
AA	39	318.20±88.22			246.94±92.88			59.22±20.52		

*One way ANOVA test, P<0.05 considered as statistically significant. Data are shown as mean ± SD.

Serum IL-21 showed higher level in AA genotypes compared with CC and CA genotype (p = < 0.001) and CA genotype has higher serum IL-21 level than CC genotype(p=0.041). The result of serum Anti-CCP showed higher levels in AA genotype compared with CC and CA genotypes (p = < 0.001 and 0.003 respectively) while there was no statistical difference between CC and CA genotypes (p = 0.689). For serum RF, AA and CA genotypes showed higher levels compared with CC genotype (p =0.002 and 0.032 respectively) while there was no statistical difference between AA and CA genotypes. For IL-21 (rs2221903) variants, there were no statistical differences of those parameters between different genotypes (data not shown).

Table 4: Two group comparison between different genotypes of IL-21 rs2055979 with respect to serum levels of Anti-CCP, RF and IL-21 and DAS28-ESR in subjects with RA

IL-21(rs2055979) Genotype	DAS28-ESR *P*	IL-21 *P*	Anti-CCP *P*	RF *P*
CC vs CA	0.638	0.041*	0.689	0.032*
CC vs AA	0.01*	< 0.001*	<0.001*	0.002*
CA vs AA	0.064	< 0.001*	0.003*	0.181

*Student t- test or Man Whitney tests, *P<0.05 considered as statistically significant. Data are shown as mean ± SD.

Discussion

RA is an inflammatory autoimmune systemic disorder caused by a series of pathological events leading to joint erosion and destruction [23], [24].

The etiology of RA is still uncertain. Both genetic and environmental factors share in its development however, the genetic factor may be considered the principal risk factor for RA [25], [26]. Cytokines such as tumor necrosis factor α and interleukins like IL-1β, IL-17 and IL-21 are some of the key mediators of RA [27]. Recently, IL-21 and IL-21R have been found to be key players in the pathogenesis of RA (28],[29],[30]. It was reported that the main cells expressing IL-21R are CD4+ T cells, macrophages, dendritic cells and synovial fibroblasts in RA subjects [31]. IL-21 is particularly produced by Th17, T (Tfh) and NKT cells and promotes the activation of NK cells, B cells and the antibodies production. The recognition and response of IL-21R to IL-21 occurs through MAPK, PI3K/AKT, and JAK-STAT pathways [32].

Furthermore, SNPs in IL-21 and its receptor were investigated in several autoimmune diseases such as multiple sclerosis, autoimmune thyroid diseases, systemic lupus erythematosus (SLE), and RA in various ethnicities [10], [11], [12], [13].

However, the role of IL-21 in RA pathogenesis has never been critically studied in Egyptian population. In the present research, we examined IL-21 (rs2055979 and rs2221903) polymorphisms and IL-21 serum level in a group of Egyptians with RA and a group of healthy subjects. Regarding IL-21 (rs2055979) polymorphism, the AA genotype and A allele were higher in subjects with RA compared with the controls. Similar results were previously obtained in several ethnicities such as Mexican populations [14] and Chinese population [15]. For IL-21 (rs2221903) variants, we did not find association between these variants and RA risk. These results coincide with studies in other populations such as Chinese [15] and Mexican populations [14] however one study in Egypt reported association between IL-21 (rs2221903) TT genotype and T allele with RA risk [19]. This discrepancy could be explained, among other factors, by differences in the genetic backgrounds of the participants, non-understandable environmental factors, small patients' sample size, patient selection, disease activity and treatment modalities.

Although the studied SNPs (IL-21 rs2055979 and rs2055979) are present in the introns and not in the coding regions (exons), they might have an important role in the regulation of gene expression through mRNA editing and control of protein translation [15], [33].

Furthermore, we analyzed the association between the SNPs and the disease activity; by determining DAS28-ESR score. We found that DAS28-ESR is higher in AA genotype of IL-21 rs2055979 than CC genotype. Similar results were obtained by other investigators [15]. However, another study obtained contrary results [14].

Meanwhile, we determined IL-21 serum level in studied groups and evaluated whether it is related to the studied SNPs. We found that serum IL-21 was higher in RA subjects in comparison with the controls confirming its critical role in RA. This result agreed with other studies in different population such as Mexican [34] and Chinese populations [15]. The presence of high IL-21 levels in autoimmune conditions, and its relationship to RA clinical signs and autoantibodies, suggests a regulatory function via IL-21/IL-21R [9]. It has been reported that IL-21 stimulates proliferation of B-cell and differentiation of plasma cells which is critical for production of antibodies [35]. In this case, the SNP could be favoring the production of antibodies; however, an unambiguous relationship is not definite.

Additionally, previous reports on SLE showed higher IL-21 levels than the controls in different ethnicities such as Chinese [36], Mexican [37] and Egyptian populations [38]. This information strongly suggested a possible role of IL-21in the pathogenesis of these autoimmune illnesses.

On comparing IL-21 level between different genotypes of IL-21 (rs2055979), we found that the AA genotype has higher level than CC and CA genotypes. Meanwhile, other inflammatory markers (ACCP and RF) were also higher in the AA genotype than CC genotype. Similar results were obtained in other ethnicities [15]. Additionally, elevated IL-21 level was

reported in SLE patients with AA and CA genotypes [38]. Nevertheless, controversial results were obtained by other research that showed that there is no statistical difference in serum IL-21 level between patients with recent RA onset and healthy subjects [39]. Explanation may be referred to differences in genetic background and presence of various environmental variables.

Our results confirmed that the IL-21 (rs2055979) AA genotype and A allele confer increased susceptibility to RA than CC genotype and C allele while no association with IL-21 (rs2221903).

The current research has certain limitations due to the small number of participants. More research is required in a larger RA cohort with clinical data and other related cytokines. These studies will be important in explaining the involvement of IL-21 in the pathogenesis of RA in a specific geographic and ethnic location and in its recruitment in RA therapeutics.

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