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Original article

A study of the Association between Plasma Interleukin-21 Gene Polymorphism and the Susceptibility to Rheumatoid Arthritis in Egyptian Patients

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Abstract:

Background: Rheumatoid arthritis (RA) is a chronic, autoimmune inflammatory illness. Proinflammatory cytokines play a crucial role in the development of RA by inducing an inflammatory response. One of these cytokines is interleukin-21 (IL-21), which regulates B-cell proliferation, plasma differentiation, and immunoglobulin synthesis; consequently, IL-21's actions on B cells may take part in the development of autoimmune disorders. **Aim of the study:** to investigate the association between plasma IL-21 gene polymorphism and susceptibility to RA in Egyptian patients and to find out its relation to disease activity. **Methods:** The case-control study was performed on 100 subjects; divided into two groups: Group(I):70 RA patients, Group (II):30 healthy matched controls. Through history taking, clinical examination and assessment of IL-21 gene rs2055979 by quantitative Real-Time PCR (qRT-PCR). Evaluation of disease activity by using the Disease Activity Score (DAS-28 score). **Results:** RA patients were significantly associated with a higher frequency of rs2055979 AA genotype and A allele (p= 0.023, 0.028) with risk to RA development (OR=2.759, 2.040 respectively). IL-21 genotypes AA and CA were significantly associated with higher DAS-28 when compared to the CC genotype ($p \leq 0.05$). On regression analysis, IL21 dominant models were considered independent risk predictors of higher RA disease activity (<0.001). **Conclusion:** In the Egyptian population, IL-21 gene variants are linked to RA susceptibility. The SNP rs2055979 AA genotype was associated with Egyptian RA patients.

Keywords: RA; IL-21 gene; autoimmune disease; polymorphisms.

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Introduction

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory autoimmune disease with unknown etiology characterized by joint targeting, reduced function and gradual impairment. Synovial inflammation and proliferation explain the cardinal signs of this illness, which include pain, swelling, and tenderness followed by cartilage loss, bone erosion, and later joint deformities [1,2].

Genetic investigations have revealed over 100 polymorphisms imparting disease risk, established the substantial relationship of RA with MHC class II alleles, and underlined the essential role of adaptive immunity, particularly effector T cells in RA pathogenesis [3,4].

Several loci are substantially related to RA through various methods. These loci are implicated in cytokine signaling, innate immune activation, and lymphocyte receptor activation through alterations in CD40, CD28, PTPN22, and others [5,6].

Interleukin-21 is a cytokine secreted by activated CD4+ T cells and natural killer T cells. The IL-21 receptor (IL-21R) is abundantly expressed on CD4+ T cells, dendritic cells, macrophages, and synovial fibroblasts. These immune cells sense IL-21 in the micro-environment and do a series of complicated actions [7]. IL-21 has a range of immune impacts and plays a vital role in the proliferation of B-cells, differentiation of plasma cells, and immunoglobulin synthesis; hence, IL-21's actions on B cells may have a role in the development of various autoimmune disorders [8].

The study aimed to investigate the association between plasma interleukin-21 gene polymorphism and RA susceptibility in Egyptian patients and to find out its relation to disease activity.

Subjects and Methods:

Subjects

The study was conducted as a case-control study from November 2021 to December 2022. One hundred subjects were enrolled and classified into; Group (I):70 RA patients, Group (Il):30 healthy age and sex matched controls. RA patients were recruited from the outpatient clinic and inpatient department of Rheumatology, Rehabilitation, and Physical Medicine at Benha University, and the laboratory work was done in the Clinical and Chemical Pathology Department at Benha University hospitals. The research was accepted by the Benha Faculty of Medicine's Ethics Committee No. (Ms.10-10-2021). All participants provided informed written consent for enrollment, and the process and any repercussions were thoroughly explained following the Helsinki Declaration. Inclusion criteria of patients group: they were diagnosed using the American College of Rheumatology / European League Against Rheumatism's (ACR/EULAR) 2010 categorization criteria for RA [9]. The research excluded individuals under the age of 18 and those with autoimmune diseases other than RA, as well as RA with hypothyroidism, hyperthyroidism, chronic liver failure, diabetes, and congestive heart failure. All participants underwent full history taking; thorough clinical examination and evaluation of the disease activity using the RA disease activity score (DAS-28 ESR) [10].

Sample collection.

Seven milliliters (7ml) of venous blood were obtained from each individual under strict aseptic conditions and separated into three parts:

1) 2 ml of blood was collected into an ethylene diamine tetra-acetate (EDTA) tube and then separated into 2 aliquots one was used for complete blood count (CBC) and the other kept at -70ºC for subsequent IL-21 rs2055979 polymorphism detection by real-time polymerase chain reaction (RT- PCR).

2) 1 ml was put into the Na citrate tube used for the ESR.

3) 4 ml was placed in a serum separating tube, allowed to clot at room temperature, and then centrifuged at 1500 rpm for 15

minutes to separate serum for clinical chemistry assays. On the day of sampling, laboratory assessment was done in the form of complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), liver and kidney function tests, rheumatoid factor (RF) and anti-cyclic citrullinated peptides antibodies (Anti-CCP Abs).

Methods

Lab tests included.

The CBC was performed using a Sysmex XS-500i automated cell counter from Japan. ESR was identified using the Westergren technique. CRP was identified using CRP-Latex Slide Agglutination provided by (SPINREACT) (Spain). Liver and kidney function tests were done using the Biosystems A15 auto-analyzer (Barcelona, Spain). RF was detected by the Nephelometry test, and anti-CCP Abs was detected by ELISA.

Molecular biology investigation: Determination of IL-21 gene single nucleotide polymorphism (SNP) rs2055979 genotype by (qRT-PCR). Genomic DNA was extracted from EDTA blood using an "EXTRACTME" genomic DNA &RNA extraction kit designed for rapid and efficient purification of high-quality genomic DNA from whole blood (BLIRT S. A, ul, Cat. No. EM13-050).

Amplification by Real-Time PCR

Genotyping of IL-21 gene SNP (rs2055979) was performed using the TaqMan SNP Genotyping assays (Applied Biosystems, Lithuania, Thermo Fisher SCIENTIFIC, LOT.011176979). The PCR amplification was done using the ABI-Step One Real-Time PCR instrument (Applied Biosystems, U.S.A).

In a sterile microcentrifuge tube, reagents were pipetted as follows: Use 10 µl of TaqMan®Genotyping Master Mix, 1.0 µl of TaqMan®SNP Genotyping Assay, 6.0 µl of DNase/RNase-free water, and 3.0 µl of DNA for a total volume of 20.0 µl per well.

PCR was carried out in a thermal cycler using the following heat conditions:

Pre-PCR (1 cycle) at 95˚C for 10 minutes, followed by 40 cycles of denaturation at 95˚C for 15 seconds and annealing and extension at 60˚C for a minute.

Statistical Analysis:

The obtained data was edited, coded, and tabulated using the Statistical Package for Social Science (IBM Corp., 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). Logistic regression, Ordinal regression, chi-square test, and ANOVA with post hoc tests were used.

Results

The current study included 70 RA cases, with a mean age of 46.33 years. There were 8 (11.4%) males and 62 (88.6%) females. The mean disease duration was 8.42 ± 0.70 years and the mean DAS-28 was 5.06 ± 1.12, 5.7 % of patients had low disease activity, 47.1% had moderate and 47.1% had high disease activity. Laboratory and laboratory data of RA patients are detailed in (Table 1). As regards drug therapy, 65.7% of patients were on methotrexate, 58.6% were on Leflunomide, 48.6% on hydroxychloroquine, 77.1% on steroids, and 2.9% were on biological treatment.

RA cases were substantially associated with a higher frequency of AA genotype, AA recessive model and A allele (p= 0.023, 0.038, 0.028), with risk to RA development (OR=2.759, 2.352, 2.040) (Table 2).

There were no significant associations found regarding the age and gender of RA patients, disease duration, HB, WBCs, platelets, ESR, CRP, RF, ALT and AST with IL-21 genotypes($p>0.05$ for each). No significant association between Anti-CCP and IL-21 genotypes in the patient's group was observed (p= 0.247). The mean Anti-CCP level in carriers of the CC genotype was 56.58 ± 24.24, in carriers of CA genotype 136.2 ± 33.50 and in carriers of AA genotype 69.94 ± 29.32.

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Table (1): Clinical and laboratory data of RA group.

	RA **N=70**		
	Mean±SD		
Disease duration (years)	8.42 ± 0.70		
Number of tender joints	9.10 ± 0.92		
Number of swollen joints	4.39 ± 0.51		
Disease activity score (DAS-28)	5.06 ± 1.12		
Hb (g/dl)	**Mean±SD**	11.23	±1.65
WBCs ($\times 10^3/mm^3$)	**Mean±SD**	7.26	±0.28
ESR (mm/ 1st hour)	**Mean±SD**	67.63	±2.93
Platelets($\times 10^3/mm^3$)	**Mean±SD**	285.36	±13.19
Urea (mg/dl)	**Mean±SD**	22.74	±1.37
Creatinine (mg/dl)	**Mean±SD**	0.87	±0.21
CRP titre (mg/l)	**Mean±SD**	35.51	±3.99
RF titre (IU/ml)	**Mean±SD**	73.19	±6.93
Anti-CCP-Abs titre (U/ml)	**Mean±SD**	97.94	±19.11

HB: haemoglobin, WBCs: White Blood Cells, ESR: Erythrocyte Sedimentation Rate, CRP: C-reactive Protein, RF: Rheumatoid Factor

The number of tender joints (TJ) and swollen joints (SJ) showed statistically significant differences among IL21 genotypes (p=0.010, 0.048 respectively). CA and AA genotypes were significantly associated with a higher number of TJ and SJ in comparison to the CC genotype (p=0.003, 0.036 for TJ respectively) (p=0.018, 0.041 for SJ respectively). Mean DAS-28 showed a significant difference among IL21 genotypes (p=0.002); AA and CA genotypes were significantly associated with higher DAS-28 when compared to the CC genotype (p=0.007, 0.003). At the same time, no significant variance was found between CA and AA genotypes regarding DAS-28 (p>0.05). Moreover, the stratification of studied cases according to the DAS-28 score revealed that higher disease activity was significantly associated with CA and AA genotypes (p=0.001, 0.014) (Table 3).

Ordinal Regression analysis was conducted for the prediction of RA activity; a higher number of TJ, SJ, ESR, CRP, RF, Anti-CCP and IL21 dominant models were substantially associated with the risk of higher RA activity in univariable analysis. However, in multivariate analysis, a higher number of TJ, SJ, ESR, CRP, Anti-CCP and IL21 dominant models were considered independent risk predictors of higher RA disease activity (Table 4).

Table (2): Genotypic and allelic frequencies of IL-21 rs2055979 between RA patients and the control group.

IL-21		**RA n = 70**		**Control n = 30**		**P**	**OR (95 % CI)**
		N.	**%**	**N.**	**%**	**value**	
Genotypes	**CC**	19	27.1	13	43.3	-	Reference
	CA	34	48.6	15	50.0	0.356	1.310 (0.739 – 2.321)
	AA	17	24.3	2	6.7	0.023*	2.759 (1.150 –6.621)
Dominant	**CC**	19	27.1	13	43.3	-	Reference
model	**CA+AA**	51	72.9	17	56.7	0.116	1.549 (0.898 – 2.671)
Recessive	**CC+CA**	53	75.7	28	93.3	-	Reference
model	**AA**	17	24.3	2	6.7	0.038*	2.352(1.048 – 5.275)
Alleles	**C**	72	51.4	41	68.3	-	Reference
	A	68	48.6	19	31.7	0.028*	2.040 (1.080 - 3.850)

N: number, C: cysteine, A: Arginine, OR: odds ratio, CI: confidence interval, *: p≤0.05 (significant); OR<1 is considered protective; OR>1 is considered risky.

Table (3): Comparison of DAS-28 between genotypes in the RA group.

	IL-21			**Test (p1)**	**Post hoc test**		
	CC	**CA**	**AA**				
	n = 19	**n = 34**	**n = 17**		P2	P3	P4
DAS-28							
Low	4 (21.1%)	0 (0.0%)	0 (0.0%)	X^2= 15.31, p= 0.002*	0.001*	0.014*	0.546
Moderate	12 (63.2%)	13 (38.2%)	8 (47.1%)				
High	3 (15.8%)	21 (61.8%)	9 (52.9%)				
Mean ± SD.	4.29 ± 1.20	5.32 ± 0.90	5.37 ± 1.10	ANOVA= 7.107 p=0.002*	0.003*	0.007*	0.985
Range	3.05 – 7.02	3.55 – 6.95	3.71 – 7.71				

SD: standard deviation, SE: standard error;
P1: comparison between CC, CA, and AA
P2: comparison between CC, CA
P3: comparison between CC, AA
P4: comparison between CA, AA
*: p≤0.05 (significant)

Table (4): Ordinal Regression analysis for prediction of Rheumatoid Arthritis disease activity (DAS-28)

	Univariate			**#Multivariate**		
	P	**OR**	**95% CI**	**P**	**OR**	**95% CI**
Age	0.168	0.982	0.957-1.008			
Gender	0.654	1.223	0.507-2.950			
Duration	0.597	0.987	0.942-1.035			
NTJ	0.028*	5.469	1.200-24.919	<0.001*	1.053	1.035-1.072
NSJ	<0.001*	1.376	1.202-1.577	0.045*	1.974	1.947-2.002
ESR	<0.001*	1.028	1.014-1.042	0.030*	1.003	1.001-1.007
CRP	<0.001*	1.023	1.012-1.034	0.045*	1.013	1.005-1.026
RF	0.011*	1.007	1.002-1.013	0.742	1.005	0.999-1.012
Anti-CCP	0.005*	1.005	1.001-1.008	0.023*	1.023	1.011-1.061
IL-21 dominant model	0.003*	1.875	1.243-2.829	<0.001*	1.440	1.202-1.725

OR: odds ratio, CI: confidence interval; *: Statistically significant at $p \leq 0.05$.
NTJ: number of tender joints, NSJ: number of swollen joints

Discussion

In the pathogenesis of RA, inflammatory cytokines play an important role in inducing the inflammatory response and releasing other inflammatory mediators [11]. One of these cytokines is IL-21, which has various effects on the immune system and plays an essential role in the proliferation of B cells, differentiation of plasma cells, and immunoglobulin synthesis; therefore, the effects of IL-21 on B cells may take part in the development of different autoimmune disorders [7,8]. In recent years, IL-21 has played a vital role in the pathogenesis and progression of RA [12].

Our results showed that RA cases were substantially associated with a higher frequency of AA genotype and A allele (p= 0.023, 0.028), with a risk of developing RA (OR=2.759, 2.040). Another study [13] showed that RA patients had a considerably greater prevalence of homozygous mutant (AA) of the rs2055979 polymorphism than healthy controls ($p < 0.0001$, $OR = 4.342$) and the mutant (A) allele was more common in RA patients than controls ($p < 0.0001$, $OR = 2.149$), showing a crucial genetic susceptibility component for RA development. In addition, the frequency of homozygous (AA) IL-21 rs2055979 polymorphism was higher in patients with RA than in controls in the Mexican population (p= 0.0216, OR = 1.761) and

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the allele A was more frequent in RA patients in comparison to controls (p= 0.0145, OR = 1.343) [8]. Similarly, other colleagues [14] discovered that allele A carriers have a 1.4-fold increased risk of getting SLE in the Chinese population. A study conducted on a cohort of Iraqi individuals discovered that the GG genotype/G allele of IL-21 (rs2055979) may be related to a tendency to multiple sclerosis [15].

In our results, there was no significant association as regards ESR, CRP, and RF with IL-21 genotypes in the RA group, similar to another study [7] that found; the analysis of ESR, CRP and RF with the IL-21 and IL-21R polymorphisms did not show differences between the variant genotypes.

In the present study, we found no statistically significant association between Anti-CCP and IL-21 genotypes in the patient's group (p=0.247). However, another study [8] found that carriers of the AA genotype had greater anti-CCP levels than the CA genotype. (p= 0.0296), this variance may be due to the different number of patients enrolled and different ethnicity.

The present research reported that the mean number of tender joints (TJ) was 9.10 ± 0.92, whereas the mean number of swollen joints (SJ) was 4.53 ± 0.51. A study conducted in 2019 [16] showed that the mean number of TJ was 5.15±5.37 and the mean number of SJ was 5.43±5.53. In the present work, the mean disease activity score (DAS-28) was 5.06 ± 1.12, and cases were divided into low activity 5.7 %, moderate activity 47.1% and high activity 47.1%. Another study [17] found that the disease was active (DAS-28 >3.2) in 59% of the patients and highly active (DAS-28 >5.1) in 14% but 8% were in remission (<2.6). Other colleagues [18] reported that the mean DAS-28 was 2.3 ± 0.3, 84% were in remission (score ≤2.6) and 16% had low disease activity (score ≤3.2).

In the present study, the mean DAS-28 score showed a significant difference among IL21 genotypes (p=0.002); AA and CA genotypes were significantly associated with higher DAS-28 when compared to CC genotypes. Inconsistent with our results, other colleagues [11] observed a substantial association between the IL-21 rs2055979 polymorphism and DAS-28 scores; they reported that patients with AA genotypes had higher DAS-28 than CA and CC genotypes. However, our findings disagreed with a study that took place in 2023 [8], which reported that analysis of the DAS-28 score association with the IL-21 polymorphisms revealed no differences between the genotypes. This variance may be due to different disease activity grades between ours and theirs.

limitations of the study: a larger study is essential to confirm the role of IL-21 in RA susceptibility and disease activity. Further studies can focus on anti-IL-21 antibodies as a target therapy for RA. Despite these limitations, our study has the advantage that we have excluded patients with other autoimmune diseases that may alter gene levels, which is unique and removes the confounding effects of similar diseases.

Conclusion

Genetic alterations in the IL-21 gene are associated with RA in the Egyptian population. The SNP rs2055979 AA genotype was linked to an increased risk of RA. AA and CA genotypes were associated with higher DAS-28 scores and IL-21 is considered a risk predictor of higher RA disease activity.

Conflict of interest

None of the contributors declared any conflict of interest.

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