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Study the Association of IL-21 Gene Polymorphism and Some Immunological Markers with the Risk of Rheumatoid Arthritis Incidence in a Sample of Iraqi Patients

[1]Mais B. Mohammed , [1]Jinan M. J. Al-Saffar , [2]Mohammed H. Alosami

[1]Department of Biotechnology, College of Sciences, University of Baghdad
[2] Professor of Rheumatology, College of Medicine, University of Baghdad

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Abstract: Rheumatoid arthritis (RA) is a prevalent autoimmune disorder marked by chronic inflammatory synovial joints. Throughout the last decades several autoantibody systems have been discovered that are associated with RA, anti-CCP test (which measures antibodies directed to cyclic citrullinated peptides), anti-carp test (for determination of the levels of anti-CarP antibodies in patients with RA), IL-21 and IL-22 levels. The aim of the study the association of IL-21 gene polymorphism (rs2221903) with the risk of RA. The studied group include 60 RA cases (19 males and 41 females), who diagnosed was depend on the Revised diagnostic criteria established by the American College of Rheumatology (ACR), 2010. Enzyme-linked immunosorbent assay (ELISA) has been utilized in order to estimate the IL-22, IL-21, Anti-CARP and ACPA levels in serum of studied group and result of investigations were compared with 30 healthy apparently control individuals. IL-21 gene polymorphism assessed utilizing polymerase chain reaction (PCR) and result were compared with 20 controls. The present results revealed that a higher positivity of patients sera for ACPA, Anti-CARP, IL-21 and IL-22 (308.11 ± 27.13, 3896.89 ± 343.90, 118.69 ± 13.09 and 148.70 ± 6.07) respectively in comparison with control groups (3.75 ± 0.44, 39.37 ± 5.59, 27.89 ± 3.77 and 55.86 ± 4.73) correspondingly with highly significant differences ($P < 0.001$), but no significant variation were recorded in the distribution of genotype, frequency of allele and frequency of haplotype for polymorphism of IL-21 gene between the RA and controls groups. It was concluded that found frequency of haplotype for polymorphism of IL-21 gene between the RA and controls groups.

Keywords: Anti-citrullinated peptide antibody, anti-carbamylated proteins antibody, rheumatoid Arthritis, Interleukin-22, Interleukin-21, IL-21 Polymorphism (rs2221903).

Corresponding author: (Email:jinan.jawad@sc.uobaghdad.edu.iq, mohammad_alosami@yahoo.com).

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder that leads to joint degeneration, disability, and other medical issues over time (1). The lungs, hands, eyes, heart, and even the red blood cells themselves can all be affected by RA because it is a systemic illness (2).

In RA, the body's immune system mistakenly attacks its own healthy cells, most notably the synovial membrane lining the joints, leading to inflammation (Centers for Disease

Control and Prevention, 2020). Genetic, environmental, and immunological variables all have a role in the development of rheumatoid arthritis. The unique immunological mechanisms of RA, which promote chronic inflammation and joint degradation, have recently been found to be directly implicated in a complex regulatory network, comprising a range of proinfammatory cytokines and chemokines (3).

Throughout the last decades several autoantibody systems have been discovered that are associated with RA. Common methods for diagnosing RA include the measurement of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs). Positive results for RF are linked to hostility and worse health outcomes (5). In a similar vein, ACPAs have been linked to worsening illness, disability, and radiographic progression (6). Autoantibodies against citrullinated proteins (ACCP) are mostly seen in the synovium of patients with RA .

The existence of anti-carbamylated protein (anti-CarP) antibodies has been linked to radiological damage (9), and has been widely reported in RA patients (8, 9). The serum of thirty-sex to forty-five percent of RA patients has been shown contain antibodies against carbamylated proteins (anti-CarP antibodies) (10). Anti-CarP antibodies may be influenced by a number of circumstances, but so far, they have not been confirmed(11). The presence of cyanate is necessary for the post-translational alteration of lysine to homocitrulline, which is known as carbamylation (12) .

Research has pointed to the potential function of several cytokines in the development of autoimmune disorders like RA. As a result, research into potential novel cytokines and other treatment options for RA continues. Studies have demonstrated that the cytokine IL-22 has a crucial function in the RA development (13). There is evidence that IL-22 has a function in the RA development by inducing synovial fibroblast proliferation and chemokine production (14). The T lymphocytes expressing IL-22 were more common in the blood and inflamed synovium of rheumatoid arthritis patients (15). Studies have linked elevated IL-22 levels to radiographic progression in RA (16), suggesting a pathogenic/pro-inflammatory function for IL-22 in the RA development and onset. It is unclear how IL-21 contributes to the development of RA. Increased IL-21 plasma levels are related with increased disease activity and radiographic status in RA patients (18), and elevated IL-21 levels have been seen in the synovial tissue of RA patients) (17).

Material and methods

The Rheumatology Unit's expert medical professionals made the diagnosis. Rheumatoid arthritis cases who sought treatment at the rheumatology clinic at Baghdad Teaching Hospital were analyzed. A total of sixty patients with rheumatoid arthritis (RA) between the ages of 25 and 65 were identified and participated in the research (19 males and 41 females). The results of the physical examination, X-rays, and lab testing were the basis for the diagnosis. Rheumatoid factors (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and tender and swollen joint counts, were used to make the diagnosis in accordance with the Revised diagnostic criteria defined

by the American College of Rheumatology (ACR), 2010 .

The control group comprised of 30 healthy people (11 male, 19 female) randomly recruited from the Iraqi community who had no clinical or history indication of RA or any chronic condition.

Anti-carp, Anti-CCP, IL-21, and IL-22 were measured in serum /all samples with kits based on sandwich enzyme-linked immune-sorbent assay technology (ELISA), while IL-21 gene polymorphism was analyzed with polymerase chain reaction (PCR).

Extraction of genomic DNA

Genomic DNA was extracted from blood using the ReliaPrepTM Blood gDNA Miniprep System, (Promega, USA), and kept at -20^{o}C until further use, as per the manufacturer's instructions.

PCR primers for IL-21 gene

The primer was designed according to their reference sequence (rs) in the database of NCBI (National Center for Biotechnology Information). A final concentration of 100pmol/l was achieved by dissolving the lyophilized primers in nuclease-free water. Table (1) displays information about these primers, such as their sequence and the size of the PCR product they generate.

Table (1): specific primers sequence, product size and annealing temperature of the IL-21 gene

Primer Name	Sequence 5`-3`	Annealing Temp. (°C)	Product size (bp)
rs2221903-F	TGTAAAACGACGGCCAGTGCTTCCAGACAGTGCTAAAT	60	878
rs2221903-R	CAGGAAACAGCTATGACGCAGATTGCCTCTCATAAGG		

IL-21 SNP

Cytokine gene (IL-21) was assessed for Single nucleotide polymorphism in the intron variant, which included rs*2221903* (IL-21 G>A). Analysis of data and determined sequence variation between samples of specific gene using geneious software after amplification.

PCR components

After several iterations, the optimal quantities of DNA and primers were determined, and the PCR process was optimized accordingly. Table (2) displayed the reagents and conditions required for a PCR process

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

Components of master mix	Stocks	Units	Final	Units	Volumes
					One Sample
Master Mix	Two	X	One	X	12.5
Reverse primer	Ten	µM	One	µM	One
Forward primer	Ten	µM	One	µM	One
Nuclease Free Water					8.5
DNA	Ten	ng/µl	Two	ng/µl	Two
Total volume					Twenty-five

Steps	°C	Time	Cycle
Initial Denaturation	95	Five minutes	One
Denaturation	95	Thirty seconds	Thirty
Annealing	60	Thirty seconds	
Extension	72	Thirty seconds	
Final extension	72	Seven minutes	
Hold	10	Ten minutes	One

Results and discussion

Clinical and demographical parameters of the studied Groups

The study shows that 68.3% of RA patients are female, making up the bulk of the study population. In addition, the average age of RA patients was (49.45 ± 1.38 years). Patients who smoked cigarettes were more numerous than those who did not [(36 (60.0%), 24 (40.0%)] as shown table (3).

Table (3): Demographic and clinical parameters of rheumatoid arthritis (RA) patients and controls

Parameter	RA		Control	
No.	60		30	
Gender	male	female	male	Female
	19(31.7%)	41 (68.3%)	11 (36.7%)	19 (63.3%)
Age[years] Mean ± SD	49.45 ± 1.38		49.27 ± 1.94	
Smoking	Non-smoker	smoker	Non-smoker	Smoker
	24 (40.0%)	36 (60.0%)	20 (66.7%)	10 (33.3%)

Level of Anti-Carp antibodies in the sera of studied group

Recent diagnostic marker for RA is anti-Carp was detected in the sera of RA patients' group. High anti-Carp level mean among the RA patients' sera (3896.89 ± 343.90) rather than the control group (39.37 ± 5.59). To observed the relation between studied anti-carp Abs and gender in controls and RA cases, we compared the distribution of Anti-carp Abs between male and female of patients and controls. Male RA patients were observed to have anti-carp antibodies level mean (3979.86 ± 643.08), which was more than that of female patients (3858.44 ± 411.01), but such difference was not significant (P > 0.05) table (4).

Table (4): Distributions of Males and Females according to anti-carbamylated protein (Anti-carp) Ab.

Gender	Anti-carp antibody level mean ± SE (Unit)		Probability
	Cases group	Control group	
Males	3979.86 ± 643.08	49.10 ± 14.78	P < 0.001
Females	3858.44 ± 411.01	33.75 ± 2.11	P < 0.001
Total	3896.89 ± 343.90	39.37 ± 5.59	P < 0.001
Probability	P > 0.05	P > 0.05	

Additionally, the relations between the examined Anti-carp Abs and clinical parameter (smoking) of RA and control were examined. Smokers RA patients were observed to have Anti-carp level mean (3790.99 ± 438.91), which was less than that of Non-smokers patients (4055.73 ± 562.95), but such difference was not significant. table (5).

Table (5): Distributions of Smokers and Non-smokers according to anti-carbamylated protein (Anti-carp) Abs.

Smoking status	Anti-carp antibody level mean ± SE (Unit)		Probability
	Patients group	Control group	
Smokers	3790.99 ± 438.91	52.12 ± 16.16	P < 0.001
Non-smokers	4055.73 ± 562.95	33.0 ± 1.81	P < 0.001
Total	3896.89 ± 343.90	39.37 ± 5.59	P < 0.001
Probability	P > 0.05	P < 0.05	

Level of Anti-CCP Abs in the studied group sera

Anti-CCP Antibody, a diagnostic marker for RA, was found in the sera of the research population. Table (6) displays the results of the frequency analysis performed on the RA and Control groups. The ACCP Antibodies and their relationship to gender were also examined by compared the distribution of Anti-CCP antibodies between male and female of patients and controls. Male RA patients were observed to have ACCP level mean (314.65 ± 50.74), which was more than that of female patients (305.07 ± 32.43), but such variation was not significant (P > 0.05).

Table (6): Distributions of Males and Females according to anti-Cyclic Citrullinated Peptide (ACCP) Antibodies.

Gender	Anti-CCP antibody level mean ± SE (Unit)		Probability
	Patients group	Control group	
Males	314.65 ± 50.74	4.52 ± 1.17	P < 0.001
Females	305.07 ± 32.43	3.31 ± 0.17	P < 0.001
Total	308.11 ± 27.13	3.75 ± 0.44	P < 0.001
Probability	P > 0.05	P > 0.05	

Moreover, the associations between the studied Anti-CCP antibodies and clinical parameter (smoking) of RA and control were studied. There significant increased anti-CCP antibody level mean of smokers patients compared to smokers controls and non-smokers patients compared to non-smokers controls table (7).

Table (7): Distributions of Smokers and Non-smokers according to anti-Cyclic Citrullinated Peptide (ACCP) Antibodies

Smoking status	Anti-CCP antibody level mean ± SE (Unit)		Probability
	Patients group	Control group	
Smokers	299.75 ± 34.63	4.75 ± 1.28	P < 0.001
Non-smokers	320.64 ± 44.42	3.25 ± 0.14	P < 0.001
Total	308.11 ± 27.14	3.75 ± 0.44	P < 0.001
Probability	P > 0.05	P < 0.05	

IL-22 Antibodies level in the sera of studied groups

IL-22 level was detected in the sera of RA patients, high level of IL-22 among RA patients (148.70 ± 6.07) rather than control group (55.86 ± 4.73). IL-22 level in male patients (157.88 ± 16.75) was higher than female (144.44 ± 4.43), but such increased was not significant (P > 0.05) table (8).

Table (8): Distributions of Males and Females according to Interleukin 22 (IL-22)

Gender	IL-22 level mean ± SE (Unit)		Probability
	Patients group	Control group	
Males	157.88 ± 16.75	60.14 ± 7.94	P < 0.001
Females	144.44 ± 4.43	53.38 ± 5.98	P < 0.001
Total	148.70 ± 6.07	55.86 ± 4.73	P < 0.001
Probability	P > 0.05	P > 0.05	

The correlation between IL-22 and smoking was also studied, reveals that the levels of IL-22 were raised in non-smokers of patients with RA in comparison with smokers, but such increased was not significant table (9).

Table (9): Distributions of Smokers and Non-smokers according to Interleukin 22 (IL-22)

Smoking status	IL-22 level mean ± SE (Unit)		Probability
	Patients group	Control group	
Smokers	146.24 ± 8.69	61.03 ± 10.60	P < 0.001
Non-smokers	152.38 ± 7.94	53.27 ± 4.86	P < 0.001
Total	148.70 ± 6.07	55.86 ± 4.73	P < 0.001
Probability	P > 0.05	P > 0.05	

IL-21 Ab Level of 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 elevated among RA patients (118.69 ± 13.09) compared to control group (27.89 ± 3.77) and the level of IL-21 in male patients (143.53 ± 36.14) was higher than female (107.18 ± 5.58), but such increased was not significant (P > 0.05) (Table 10).

Table (10): Distributions of Males and Females according to Interleukin 21 (IL-21).

Gender	IL-21 level mean ± SE (Unit)		Probability
	Patients group	Control group	
Males	143.53 ± 36.14	32.04 ± 7.52	P < 0.05
Females	107.18 ± 5.58	25.45 ± 4.14	P < 0.001
Total	118.69 ± 13.09	27.89 ± 3.77	P < 0.001
Probability	P > 0.05	P > 0.05	

And also studied the relations between the IL-21 and smoking. There is non-significant increased IL-21 level mean of non-smokers patients (133.31 ± 24.23) compared to smokers (108.94 ± 12.03) table (11).

Table (11): Distributions of Smokers and Non-smokers according to Interleukin 21 (IL-21).

Smoking status	IL-21 level mean ± SE (Unit)		Probability
	Patients group	Control group	
Smokers	108.94 ± 12.03	33.29 ± 7.65	P < 0.05
Non-smokers	133.31 ± 24.23	25.16 ± 4.19	P < 0.001
Total	118.69 ± 12.06	27.87 ± 3.77	P < 0.001
Probability	P > 0.05	P > 0.05	

IL-21 SNPs in the studied groups

Two alleles (G, A) and three genotypes (AA, GA, and GG) were provided for the SNP (rs2221903 G/A). Genotypes were found to be in agreement with Hardy-Weinberg equilibrium (HWE) analysis in RA patients and control group, with no statistically significant discrepancies between actual and anticipated genotype frequencies (Table 12). Although the G allele was less common in RA patients (10.0 vs. 15.0%) and the A allele was more common in RA patients (90.0 vs. 85.0%) than in controls (table 13), there was no statistically significant difference between the genotype and allele frequencies of these two groups.

Table (12): Percentage frequencies and number (expected and observed) of *IL-21* gene (rs2221903SNP) genotypes and their Hardy-Weinberg equilibrium (HWE) in RA cases and control group.

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

Table 13: statistical analysis of associated between alleles and genotypes of *IL21* gene (rs2221903SNP) and RA group.

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

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

Despite the fact that the average age of onset for this disease is 65 (19), the mean age of our patients was only 49.45 ± 1.38 years. Similar findings were seen in (20). where the average age was 41.6 ± 11.7 years. Our results, which are consistent with the findings of (21), show that women make up a greater proportion of the study population when accounting for age.

According to the results of our research, cigarette smokers are more likely to get the condition than non-smokers. The findings were consistent with those of (22). conducted the first meta-analysis to examine the link between smoking and RA, finding that heavy male smokers who also test positive for RF are more likely to acquire the disease. Smokers had around a twofold higher chance of getting RA compared to non-smokers.

A prior multicenter cohort research (Sweden, n=795; United Kingdom, n=761 and Netherlands, n=678) established that smoking was related with many Abs positivity (anti-CarP Abs, anti-CCP2 Abs, and RF). However, our research showed that smoking was not linked to an increased risk of autoantibodies (23).

In our investigation, we found that the prevalence of anti-CarP antibodies was higher in RA patients than in the control group (Table 4). Similar results were seen in a study of Japanese RA patients (24) where substantial differences in anti-CarP levels were found ($p<0.001$).

In this study, individuals with RA had higher levels of ACCP than the healthy controls did (Table 6). Consistent with the results of the study established by (25), we find that the serum anti-CCP antibody level is a promising potential diagnostic indicator of RA.

According to (Table 8), IL21 levels in rheumatoid cases were significantly greater than those in healthy controls. This is consistent with the (26) finding that plasma IL-21 levels were considerably greater in RA cases (19.6 ± 0.79 ng/mL) in comparison with HC (2.12 ± 0.08 ng/mL) ($p< 0.0001$). Table 10 further shows that the mean IL22 levels of cases with RA were substantially greater than those of control subjects (148.70 ± 6.07 pg/ml vs. 55.86 ± 4.73 pg/ml, $p<0.001$). Similarly, in a research conducted by (27), controls had lower levels of IL-22 than RA patients (mean 67.45 pg/ml versus 432.37 pg/ml, respectively; $p<0.001$).

Also looked at genetic variations in IL-21-encoding genes in RA patients. Based on our findings, SNPs of IL-21 are not the genetic loci influencing to RA development. There

were no significant variations in the examined genotypes distribution among RA patients and controls (28).

Conclusion

The present results revealed that a higher positivity of patients sera for ACPA, Anti-CARP, IL-21 and IL-22 at 308.11, 3896.89, 118.69 and 148.70 respectively in comparison with control groups at 3.75, 39.37, 27.89 and 55.86 correspondingly with highly significant differences ($P < 0.001$), but no significant variation were recorded in the distribution of genotype, frequency of allele and frequency of haplotype for polymorphism of IL-21 gene between the RA and controls groups.

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