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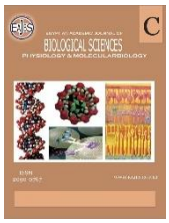
Citation: *Egypt.Acad.J.Biolog.Sci. (C.Physiology and Molecular biology) Vol. 15(2) pp391-401 (2023)*
DOI: 10.21608/EAJBSC.2023.319638

Egypt. Acad. J. Biolog. Sci., 15(2):391-401 (2023)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
www.eajbsc.journals.ekb.eg



Association of IL-17A (rs2275913) G/A and IL-21 (rs4833837) C/T Gene Polymorphisms in Iraqi patients with Rheumatoid Arthritis

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ARTICLE INFO

Article History
Received: 24/8/2023
Accepted: 3/10/2023
Available: 7/10/2023

Keywords:
Rheumatoid arthritis, IL17 (rs2275913) G/A, IL-21 (rs4833837) C/T, Tetra ARMS- PCR, Allele-specific PCR.

ABSTRACT

Rheumatoid arthritis is a persistent autoimmune disorder that causes both systemic and local inflammation. Several proinflammatory cytokines, such as interleukin (17A)and interleukin (21), genetic variation, and environmental variables induce it. The purpose of this study is to examine IL-17A (rs2275913) G/A and IL-21 (rs4833837) C/T gene polymorphism association in Iraqi patients with rheumatoid arthritis. The current study includes 120 subjects classified into two main groups: 60 RA patients and 60 healthy controls. Tetra ARMS- PCR and Allele-specific PCR were used for the detection of IL-17A and IL-21 gene polymorphisms respectively. Genotype and allele frequency results of IL17 rs2275913 G/A and IL-21 rs4833837 C/T gene polymorphisms were analyzed under codominant, dominant, recessive, and additive models. IL17A (rs2275913) G/A under codominant pattern with heterozygous genotype (G/A) and dominant pattern, those of the GA+AA genotypes significantly higher in RA patients with respect to those of the control group (OR:3.3, CI 95%: (1.11 to 9.96), P< 0.03) and (OR: 3.35, CI 95%: (1.12 to 9.99), P< 0.03) respectively. In addition, this genetic polymorphism doesn't appear significant variation in other inheritance models. The allele (A) frequency was observed to be higher in RA patients (49%) opposite the allele (G) frequency observed to be higher in control (59%) without significant variation between them, On the other hand, IL-21 rs4833837 C/T genotyping and allele frequencies results revealed no significant variation between the rheumatoid arthritis patients in comparison with the control groups.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder that typically affects tiny joints and has a long-lasting, systematic etiology. It's characterized by inflammation in the synovial membrane and cellular growth, which can degrade cartilage and bone over time Joint discomfort, fever, and fatigue are all symptoms of this condition (Roy and Ghosh,2013). Symmetrical impacts on the wrists and hands are the most common, although other areas and organs can be affected as well (Guo *et al.*, 2018). Rheumatoid arthritis-related synovitis is characterized by inflammation and a thickening of the synovial tissue. It also affects the underlying cartilage and bone. In many cases, the onset of illness takes place over a period of weeks or months (Rajitha *et al.*, 2017) Tissue damage and chronic inflammation in RA joints have been linked to the production of Th17-specific cytokines such as interleukin 17 (IL-17), interleukin 21 (IL-21), and interleukin 22 (Kamoona and aljanaby, 2023).

The diseased synovium produces more inflammatory mediators when exposed to IL-17, a proinflammatory cytokine. Synergistic enhancement of these mediators is seen when IL-17 is combined with additional cytokines as TNFa, IL-1b, IL-6, IL-23, and G-CSF (Acosta-Rodriguez *et al.,* 2007; Chen *et al.,* 2007). Additionally, RANKL (receptor activator of NF-kB ligand) activation by IL-17 causes bone deterioration in inflammatory joints by promoting osteoclastogenesis (Bogunia-Kubik *et al.,*2015). Numerous studies have revealed that RA patients had higher IL-17 levels than healthy individuals in their synovial fluid, synovium, and peripheral blood mononuclear cells. (Li *et al.,* 2012). Genetic polymorphisms of pro-inflammatory cytokines and cytokine receptor genes, chemokines and their receptors, key intracellular signaling pathway components, and auxiliary (costimulatory) factors play significant roles in RA susceptibility and disease progression (Mikhaylenko *et al.,* 2020). IL-17A rs2275913 and IL-17F rs763780 polymorphisms are significantly associated with susceptibility to RA (Bogunia-Kubik *et al.,* 2015). Changes in the CCR6 gene can influence the progression of RA. This gene genes for the chemokine receptor, which is found on the surface of Th17 cells. The CCR6 50 regulatory sequence dinucleotide polymorphism rs3093024 binds nuclear proteins. This polymorphism is linked to chemokine receptor expression and elevated plasma concentrations of the cytokine IL17 in RA patients.(Cheng et al., 2015; Messemaker et al., 2015). Interleukin-21 (IL-21) cytokine is a member of the IL-2 family mainly produced by CD4 + T cells and natural killer T cells (NKT) (Spolski & Leonard, 2014). IL-21 plays a vital role in the regulation of both innate and adaptive immune systems(John *et al.,* 2010). Notably, IL-21 regulates Th17 cell development, B cell activation, and immunoglobulin synthesis. (Niu *et al.,* 2010). In the Chinese population, the IL-21 rs2055979 polymorphism is related to IL-21 plasma levels and is predisposed to RA development. (Hao *et al.,* 2021) (Liu *et al.,* 2021). The IL-21 rs2221903 polymorphism is associated with disease activity(Malinowski *et al.,* 2017). The rs6822844 SNP, which is found in the intergenic region between the IL21 and IL2 genes, has the highest connection with RA susceptibility.(Ren *et al.,* 2019).

MATERIALS AND METHODS

1.Patient and Control:

-Patient group, a total of 60 cases of female Rheumatoid arthritis were examined during the period between January/ 2022 to April/ 2022. The age of RA individuals is between 24 and 75 years. Arthritis patients were attending in Merjan Teaching Hospital, Rheumatology Unit Babylon province and Al-sader Teaching Hospital, Medical Rehabilitation and Joint Unit, Al-Najaf Al-Ashraf province and examined by specialist physicians and diagnosed as rheumatoid arthritis patients depending on the clinical and serological parameters according to 2010 ACR/ EULAR criteria.

- **Control group,** composed of 60 females apparently healthy persons. The age of the control persons is between 24 and 70 years. The samples were taken from persons who did not exhibit RA symptoms and had no history of autoimmune diseases during the clinical and serological examination

2.Blood Samples:

Blood from patients and control groups was collected and transferred into two tubes, (1 ml) transferred to EDTA tube in order to the extraction of DNA then amplification by PCR for the study of miR-146a C/G SNP and (3 ml) blood was transferred to gel tube and centrifuged at 4000 rpm for 5 minutes to separate serum then the serum was frozen at -20 ºC for measurement of clinical parameters.

3. Sequence of Primers:

The sequence of primers were used in this study were mentioned in Table (1).

Table 1: Sequence of primers used in this study.

Primer	Direction	Sequence	Product
IL-17A rs2275913 G/A SNP	Outer Forward	5'AATGGAAAATCAAGGTACATGACACC-3'	404bp
	Outer Reverse	5'GATGGATGAGTTTGTGCCTGCT-3'	
	Inner Forward (G allele)	5'TTCCCATTTTCCTTCAGACGG-3'	193bp
	Inner Reverse (A allele)	5'-CCCCAATGAGGTCATAGAAGAATCTATT-3'	260bp
IL-21 rs4833837 C/T SNP	Left1 primer (C allele)	5'-TTA GTT GCG CCT TCT GAA Aa-3'	156pb
	Left 2 primer (T allele)	5'-TTA GTT GGG CCT TCT GAA Ag-3'	
	Right primer	5'-TGT AAT GCA CGA CAT TGC AG-3'	

3. DNA Extraction and Amplification:

- **DNA Extraction and Purification:**DNA extraction FavorPrep^{TM} Blood/ Cultured Cells Genomic DNA Extraction Mini Kit (FAVORGEN-TIWAN) is used for DNA extraction from whole blood. The purity of human DNA was estimated by UV/Visible spectrophotometer at 260/280 nm and according to the instruction of extraction kit the accepted absorbance ratio for pure DNA ranges between 1.7 and 2.1 to give DNA yields about 4-10 µg/ml.
- **DNA Amplification:**GoTaq® Green Master Mix (Promega-USA) components, volumes and their concentrations for amplification of IL-17 G and/or A allele (s **)** for SNP detection by ARMS-PCR were reported in Table (2-A). IL21 C and/or T allele(s) for SNP detection by Allele-specific PCR-PCR were reported in Table (2-B). PCR Program for both IL17 G/A and IL21 C/T SNP detection were detailed in Table (3**)**The PCR products were analyzed by agarose gel electrophoresis at 75 volts, for 80 minutes. Agarose gel was positioned on the UV trans-illuminator of gel documentation under UV beam and the pictures were taken by means of a camera.

Table (2-A): Mixture of ARMS-PCR for IL-17 G/A SNP detection.

Mixture solution	Volume	Concentration
Master mix	12.5 µl	1X
Target DNA	5 µl	-
Forward Outer Primer	1.0 µl	10 pmol/ µl
Reverse Outer Primer	1.0 µl	10 pmol/ µl
Forward Inner Primer	1.5 µl	10 pmol/ µl
Reverse Inner Primer	1.5µl	10 pmol/ µl
Nuclease free water	2.5 µl	-
Total volume	25 µl	-

Table (2 -B): Mixture of Allele Specific-PCR for IL-21 C/T SNP detection.

Mixture solution	Volume	Concentration
Master mix	12.5 µl	1X
Target DNA	5 µl	-
Forward-1 primer (T allele) for each tube added only with a reverse primer	1.5 µl	10 pmol/ µl
Forward-2 primer (C allele) for each tube added only with reverse primer	1.5 µl	10 pmol/ µl
Reverse primer was added for each forward-1 tube mixture and forward-2 tube mixture	1.5 µl	10 pmol/ µl
Nuclease free water	4.5 µl	-
Total volume	25 µl	-

Table 3: Amplification conditions of IL-17 G/A SNP and IL-21 C/T SNP**.**

Steps	Temperature	Time	No. of cycle
Initial denaturation	95 ºC	5 minute	1
Denaturation	95 ºC	30 second	35
Annealing	60 ºC	30 second	
Elongation	72 ºC	30 second	
Final elongation	72 ºC	5 minute	1
Hold	4 ºC	30minute	1

4.Statistical Analysis:

Hardy–Weinberg equilibrium (HWE) HWE can be illustrated arithmetically:

p2+2pq+q2 = 1 Where ‘p’ and ‘q’ represent the frequencies of alleles.

The multi-nominal logistic regression was estimated by IBM SPSS version 23 to evaluate the relationship between genotype and the frequencies of allele in rs2910164 C/G MiRNA146a. The output data was analyzed as (OR), (CI 95%) and P value. The level of significance that was used was ≤ 0.05 in all statistical analysis and this type of statistical analysis were used according to (Shahid et al., 2013).

RESULTS

1.Detection of IL-17A (rs2275913) SNP:

Tetra-primers for the amplified products of IL-17A(rs2275913) gene by T-ARMS-PCR (Mohsen *et al.*,2020). Forward outer(FO) and reverse outer (RO) primer sets give the amplified product of 404 bp as an internal control. Forward inner (Allele-G) and Reverse outer primer set makes the product size of 193 bp for allele-G and reverse inner (Allele A) and forward outer primer pair gives a 260 bp product for allele A. Heterozygous GA genotype showed when primer pair of allele-G (forward inner) and (RO) primers amplify the allele G and; primer pair allele-A (reverse inner) and (FO) amplify the allele A with outer internal control (F,R) resulting three bands (404bp,260bp,193bp). In homozygous GG genotype pattern, primer set of allele-G (forward inner) and (RO) amplify the product of allele-G and control DNA fragment resulting in two bands (404bp,193bp). In homozygous genotype AA genotype primer set of allele-A (reverse inner) and (FO) amplifies the allele A and control DNA fragments give two bands(404bp,260bp) as shown in Figure (1).

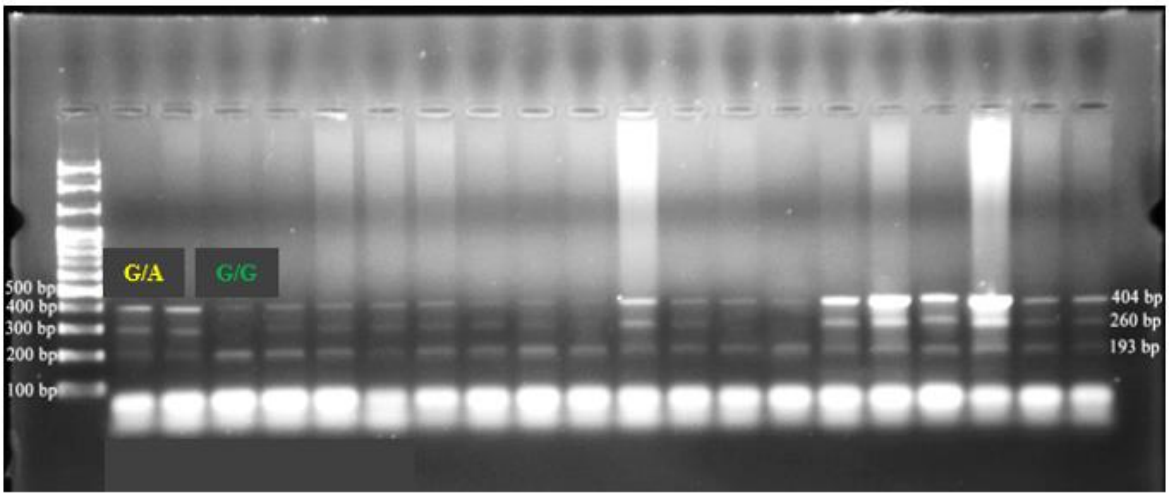

Fig. 1. Agarose gel electrophoresis image of ARMS- PCR products Detection of IL-17A rs2275913 SNP. M: marker (100bp). Agarose gel (1.5% agarose gel stained with ethidium bromide 80 min, 75 Volt, 1X TBE buffer**.**

2.Detection of IL-21 rs4833837 C/T SNP:

Allele-specific PCR(ASP) was used to detect the product of IL-21 rs4833837 gene which used three primers right primer and two left primers (L1 for amplifying allele C and L2 for amplifying allele T) resulting in amplicon 156pb (Kamoona and Aljanaby, 2023). A Heterozygous C/T genotype pattern resulted when a set of right and two (L1 and L2)primers were used to amplify C allele and T allele. Homozygous C/C genotype pattern resulted when the primer pair of right and (L1) were used to amplify allele C. Homozygous T/T genotype pattern resulted when the primer pair of right and (L2) were used to amplify allele T. Figure 2 illustrated the results.

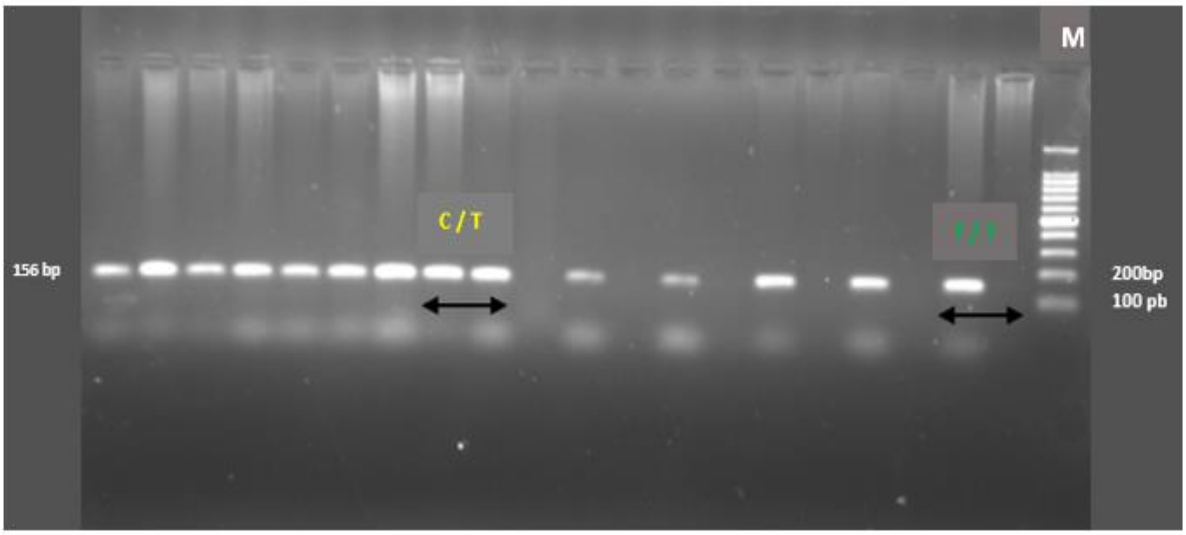

Fig. 2: Agarose gel electrophoresis image of ARMS- PCR products Detection of IL-21 rs2275913 SNP(156bp). M: marker (100bp). Electrophoresis conditions: Agarose gel (1.5% agarose gel stained with ethidium bromide 80 min, 75 Volt, 1X TBE buffer).

3.Estimation of Genotype And Allele Frequencies of IL17 rs2275913 G/A Gene Polymorphisms:

Genotype and allele frequency results of IL17 rs2275913 G/A gene polymorphisms were examined by

multinomial logistic regression, under codominant, dominant, recessive, and additive models in RA patients and the healthy subjects illustrated in Table (4). When the co-dominant model is used, the RA patients with heterozygous genotype (G/A) seem to be a significant variation in the comparison with those of the wild type of the control group (OR:3.3, CI 95%: (1.11 to 9.96), P< 0.03) higher in RA patients with respect to those of the control group. However, such variation is evidently changed when the analysis is directed towards the homozygous genotype (A/A) variant (OR: 3.73, CI 95(0.61 to 22.86), P< 0.15). Under the dominant pattern, those of the GA+AA genotypes, were indicated to be significant (OR: 3.35, CI 95%: (1.12 to 9.99), P< 0.03) higher in RA patients with respect to those of the control group. The recessive model was found to exhibit no significant variation (OR: 0.74, CI 95%: (0.16 to 3.44), P< 0.69). The allele (A) frequency was observed to be higher in RA patients(49%) opposite the allele (G) frequency observed to be higher in control group as shown in Table (5).

Table 4: Genotype of rs2275913 G/A polymorphism for IL17 gene of total healthy subjects and total patients group.

rs2275913 G/A IL17	Control N=60	Patients N=60	OR (CI 95%)	Adjusted OR P value
Codominant				
G/G	14	5	1.0	
G/A	43	51	3.3 (1.11 to 9.96)	0.03
A/A	3	4	3.73 (0.61 to 22.86)	0.15
Dominant				
G/A+A/A	46	55	3.35 (1.12 to 9.99)	0.03
G/G	14	5	1.0	
Recessive				
G/G+G/A	57	56	1.0	
A/A	3	4	0.74 (0.16 to 3.44)	0.69
Additive				
G	71	61	1.0	
A	49	59	0.7 (0.43 to 1.18)	0.19

Table 5: IL-17 allele frequencies.

IL17 allele frequencies (n=120)						
	All subjects		Group=Control		Group=patient	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	132	0.55	71	0.59	61	0.51
A	108	0.45	49	0.41	59	0.49

4.Estimation of Genotype And Allele Frequencies of IL-21 rs4833837 C/T Gene Polymorphisms:

Genotype and allele frequency of IL-21 rs4833837 C/T gene polymorphisms results were examined by multinomial logistic regression, under codominant, dominant, recessive, and additive models in RA patients and the healthy subjects illustrated in Table (6).

The results revealed no significant variation between the RA patients in the comparison with the control group under all codominant model heterozygous genotype (C/T), codominant model homozygous genotype (T/T), dominant pattern(C/T+T/T) genotypes and recessive model (T/T) genotype. Analysis of the allele frequencies no statistically significant differences between patients and controls as shown in Table (7).

Table 6: Genotype of rs4833837 C/T polymorphism for IL-21 gene of total healthy subjects and total patients group.

rs4833837 C/T IL21	Control N=60	Patients N=60	OR (CI 95%)	Adjusted OR P value
Codominant				
C/C	3	0	1.0	
C/T	50	5	7.3 (0.37 to 144.47)	0.19
T/T	7	8	7.9 (0.35 to 179.97)	0.19
Dominant				
C/T+T/T	57	60	7.4 (0.37 to 145.75)	0.18
C/C	3	1	1.0	
Recessive				
C/C+C/T	53	52	1.0	
T/T	7	8	1.2 (0.39 to 3.44)	0.78
Additive				
C	56	52	1.0	
T	64	68	1.14 (0.68 to 1.90)	0.6

Table 7: IL-21 allele frequencies.

IL21 allele frequencies (n=120)						
	All subjects		Group=Control		Group=patient	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
T	132	0.55	64	0.53	68	0.57
C	108	0.45	56	0.47	52	0.43

DISCUSSION

Interleukin-17 (IL-17), a cytokine mostly released by Th17 cells, appears to be important in the pathophysiology of rheumatoid arthritis. (Gaffen, 2009). Functional genetic variants in IL-17 and its receptor genes can change their functions qualitatively or quantitatively and are linked to a variety of inflammatory disorders. (Dhaouadi *et al.*, 2018). Genotype and allele frequency results of IL17 rs2275913 G/A were analyzed under codominant, dominant, recessive, and additive. IL17A (rs2275913) G/A under codominant pattern with heterozygous genotype (G/A) and dominant pattern, those of the GA+AA genotypes significantly higher in RA patients with respect to those of the control group (OR:3.3, CI 95%: (1.11 to 9.96), P< 0.03) and (OR: 3.35, CI 95%: (1.12 to 9.99), P< 0.03) respectively oppositely don't found significant variation in other inheritance models. The allele (A) frequency was observed to be higher in RA patients(49%)

opposite the allele (G) frequency was observed to be higher in control group(59%) without significance between them. The new findings are similar to prior research in the Pakistani population, which found that IL17A rs2275913 had a significantly different frequency solely for the dominant model of inheritance, with no significant differences reported at the allelic level. (Amin *et al.,* 2021) There is also evidence of a relationship between the IL-17A rs2275913 polymorphism and the pathophysiology of RA in the Korean population. (Lee & Bae, 2017) . Consistent with current findings, the rs2275913 polymorphism of the IL17A gene is associated with an increased risk of RA and a more severe disease in Egyptian RA patients; however, RA patients had a higher frequency of the rs2275913 G allele than healthy subjects (P = 0.01).(Ibrahim *et al.,* 2023). According to previous meta-analyses, the rs2275913 G allele increased the incidence of RA in Caucasians (OR = 1.14, 95% CI = 1.00-1.29, P =.044) but not in Mongolians (P >.05) (Shao *et al.,* 2021). The relationship between IL-17A rs2275913 and IL-17F rs763780 with the risk of osteoarthritis in a Chinese population is being investigated. (Bai *et al.,* 2019) This results disagree with (Marwa *et al.,* 2017) who found that *IL17A*-G/A polymorphism did not show any significant association with RA prevalence in the Tunisian population. (Lee & Bae, 2017), In other study by(Eskandari-Nasab et al., 2017) during meta-analysis results showed that IL-17A rs2275913 SNP was not associated with the risk of RA under codominant, dominant, and recessive models (P > 0.05). IL-21 is one of the newly investigated cytokines in the pathogenesis of autoimmune diseases (Kamoona and Aljanaby, 2023). IL-21 is an inflammatory cytokine known for its role in activating both humoral and cell-mediated immune responses, most notably the differentiation of B cells to plasma cells (Moens & Tangye, 2014).. furthermore, some genetic variations in the IL-21 gene were associated with susceptibility to SLE (Qi *et al.,* 2015). Zhou,et al.,(2015) and Zhang *et al.,(*2013) found associations of certain haplotypes containing rs4833837 with psoriasis and Hashimoto's thyroiditis (Zhang *et al.,* 2013; Zhou *et al.,* 2015). Firstly IL-21 rs4833837 C/T gene polymorphism was evaluated with RA susceptibility our results revealed not significant variation between the RA patients in the comparison with the control group under all codominant model heterozygous genotype (C/T), codominant model homozygous genotype (T/T), dominant pattern(C/T+T/T) genotypes and recessive model (T/T) genotype .Analysis of the allele frequencies no statistically significant differences between patients and controls. cytokine gene polymorphism may affect their transcription, influence their level of production and may be implicated in inducing susceptibility or resistant to diseases.

Conclusion

There is a significant association between IL-17A rs2275913 G/A gene polymorphism and risk of RA but IL-21 rs4833837 C/T gene polymorphism doesn't have an association with RA susceptibility.

Acknowledgments

Special thanks go to the staff members of Marjan Teaching Hospital (Babylon), especially to the Rheumatology Unit staff and Medical Rehabilitation and Joint Unit staph in Al-Sader Teaching Hospital/ Al-Najaf Al-Ashraf province. our indebted to (RA) patients & healthy donors. Also special thanks to the staff of the Al-Ameen Foundation for research and studies.

Interest Conflicts:

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Financial Support And Sponsorship: Not found.

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