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Role Investigation of Interleukin- IL-17 *rs763780 T/C* Gene Polymorphism with Iraqi Rheumatoid Arthritis Patients

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Abstract: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial hyperplasia, cartilage damage, and bone erosions. Rheumatoid arthritis occurs in about 5 per 1000 people and can lead to severe joint damage and disability. The clinical manifestations of symmetrical joint involvement include arthralgia, swelling, redness, and even limiting the range of motion. This study aimed to investigate the role of IL-17F rs763680 T\C gene polymorphisms that are associated with rheumatoid arthritis in a sample of Iraqi patients. In this study, there were 100 subjects participated, about 50 of them with rheumatoid arthritis (RA) so, represented patient groups, and the other 50 were healthy and represented the control groups, who were diagnosed in the Rheumatology Unit of AL-Hindyia General Hospital in Karbala province. DNA was extracted, then the genotyping polymorphism (rs763680) of the gene Interleukin-17 was done by TaqMan RT-PCR SNPs genotyping method. The genotyping and allele frequencies of IL-17 rs763680 T/C for the two groups appeared that were no significant differences in genotype between patients and controls. Compared TC genotype between control and patients, the heterozygous TC genotype was not significantly different from controls (X^2=0.601, OR=0.593), and the TT genotype were no significant differences for RA (X^2=0.538, OR=1). In addition, allele frequency for the C allele were no significant differences of allele frequencies of IL-17 rs763680 TC gene polymorphism with the risk of rheumatoid arthritis in a sample of Iraqi patients. Moreover, the association between the serum IL-17 level and IL-17 rs763680 TC genotype, in patients with RA significantly increased (P<0.01).

Keywords: Rheumatoid arthritis (RA), genetic polymorphism, ACCP, and IL-17 level.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder indicated by chronic polyarthritis and synovitis that results in joint damage. It typically affects the peripheral synovial joints symmetrically. The clinical course of the disease is prolonged and accompanied by systemic complaints (1) The manifestations are varied and early intensive management is needed to prevent joint damage and physical handicap. The disease had been described by Landre Beauvais in 1800 and the terminology of RA was first introduced by Garrod in 1859 (2).

The main symptoms of RA are joint pain, stiffness, swelling, fatigue, weight loss, and low-grade fever (3). RA may also cause issues with the heart, eyes, nerves, and respiratory system, this illness first affects synovial joints, causing discomfort, deformity, and functional impairment, resulting in significant morbidity and increased death (4).

The etiology of RA is still unknown despite advances in the understanding

of its pathogenesis. Several aspects that are also suspected of having roles in the pathogenesis of RA are as follows: genetic factor or specific gene polymorphism, pathogenic immune response and infectious agent-induced inflammation, autoimmunity toward synovium and cartilage components, interference of regulation of proinflammatory cytokines production and transformation of constituent cells in the synovial into tissue invasive, autonomous cells (5).

The perception that RA pathogenesis is a multifactorial process that needs a combination of genetic, environmental, and immune factors, has been accepted by most experts. However, the understanding of how the components are correlated with each other is still unclear. There is a possibility that those factors have roles as risk factors. They, combined with other factors, can cause the disease through mechanisms and pathways that are not yet fully understood (6).

According to that, cytokines have an essential role in the etiopathogenesis of RA and are probably responsible for inflammatory reactions and joint destruction during disease. The regulation of cytokines is unbalanced; this involves both insufficient productions of inhibitory cytokines and augmented production of proinflammatory cytokines that together contribute to the chronic inflammatory condition (7) The synovial membrane in RA contains activated T cells and B cells, macrophages, and plasmocytes. It is also known that host tissue cells, such as synoviocytes, chondrocytes, and osteoclasts, are also involved in mediating bone and joint cartilage damage. The recruitment process, activation, and effectors of each contributor mentioned earlier are regulated through a network of cytokines (8).

Interleukin-17 is a proinflammatory cytokine, produced by Th17 cells, and has pleiotropic effects on various cells contributing to the pathogenic condition of RA. Several studies showed that this cytokine maintains inflammation and causes more destruction of joint cartilage. Advances in the understanding of the role of IL-17 elicit the idea to modulate IL-17 and/or Th17 cells as the potential targets of therapy in RA (9).

Moreover, several single-nucleotide polymorphism (SNP) studies are widely considered by research and their results to autoimmune diseases. Ibrahim and Al-Tae, (2021) reported that there is a strong relationship between interleukin – 6 serum level and single nucleotide gene 174 G/C promoter polymorphism in patients with RA in a sample of the Iraqi population (10). It is concluded that the concentration of serum IL-6 was elevated in RA regarding healthy controls which confirmed its pivotal role in RA pathogenesis, while interleukin-17 rs2275913 G/A genotype had an association with osteoporosis in Iraqi pre and postmenopausal women, also the IL-17 level plays a vital role in the development risk of osteoporosis and osteopenia in pre and postmenopausal women (11).

Materials and methods

One hundred volunteers were taken in this study. 50 RA-conducted patients and 50 healthy controls, who were randomly selected between November 2021-February 2022 were diagnosed in the Rheumatology Unit of AL-Hindyia General Hospital in Karbala province. A questionnaire has been taken from the patients, and the case sheet included

age, gender, residence, height, weight, and previous history of the disease.

Five ml of peripheral blood from all select subjects through vein puncture by using disposable plastic syringes. Each blood sample was divided into two (2) ml was placed into EDTA tubes and the remaining three (3) ml were pushed slowly into a gel tube. The blood samples were placed in a cool - box under aseptic conditions and transferred to the laboratory.

The serum ACCP and IL-17 levels were measured by the ELISA sandwich technique by using a Sunlung kit (China).

According to the manufactures instructions, total genomic DNA was isolated from 200µl of whole blood by using Addbio Genomic Kit (Korea). In brief, about 200 µL of whole blood samples were lysed with lysing solution with proteinase K at 56 °C for 10 min.

The lysed cells were loaded in the DNA isolation column and centrifuged at 13,000 rpm g for 1 min. Subsequently, the column was washed twice with wash buffer. The membrane-bound DNA was eluted with elution buffer after centrifugation at 13,000 g for 1 min. The isolated genomic DNA was stored at − 20 degrees until further use. DNA concentration was estimated by using Nanodrop. Genotyping of polymorphism rs763780 of the IL-17 gene was done, by using TaqMan SNP Genotyping Assays.

A set of primers and probes was used to amplify specific regions within the IL-17 gene. As shown in table (1).

Table (1): A set of IL-17 F rs763780 T/C primer and probe genotype

Primer and Probe	Sequence	Net Count	PCR Size
Forward primer	5`- GCTGAGTGGATATGCACCTC-3`	20	110
Reverse primer	5`-CAAGGCTGCTCTGTTTCTTTC-3`	21	
Fam-probe	5`-ACTGCACACGGTGGAT-3`		
Hex-probe	5`-TACTGCACATGGTGGATG -3`		

The thermal cycling program was as follows in the table (2).

Table (2): RT-PCR cycling program

Steps	Tem. (°C)	Time (m:s)	Cycle
Carryover prevention	50	2:00	Hold
Initial Denaturation	95	10:00	1
Denature	95	0:30	39
Annealing	60	1	

Statistical analysis

The statistical analysis system-SAS (2018) program was used to detect the effect of different factors on study parameters. T-test and LSD test was used to significantly compare between means. The Chi-square test was used to significantly compare percentages (0.05 and 0.01 probability). Estimate of Odd ratio in this study. SAS. 2018. Statistical Analysis System, User's Guide. Statistical. Version 9.6th ed. SAS. Inst. Inc. Cary. N.C. The USA.

Results and discussion

Distribution of RA patients and control group according to Anti-cyclic citrullinated peptide antibody (U/ml).

The distribution of rheumatoid arthritis patients and as well as healthy control group was studied according to

ACCP (Table 2). The results showed there was a significant association between the two study groups according to ACCPA level (P<0.01)

Table (2): Distribution of rheumatoid arthritis patients and control group according to anti-cyclic citrullinated peptide antibody

Subjects	Mean ± Std error	T-test	P-Value
ACCP Patients	10.670 ± 0.901	4.031**	0.001**
ACCP Controls	6.708 ± 0.390		
* (P≤0.05), ** (P≤0.01).			

****T-test at significant 0.05.**

The ACCP is considered very helpful during the diagnosis of RA. However, anti-CCP shows a superior specificity than RF for the diagnosis of RA (17). The result of this study agreed with a different previous study that concluded a higher level of Anti-CCP in the patient group compared to the control group with higher specificity for RA compared to another inflammatory marker such as RF and CRP, which is consistent with the previous researches (12), and this result agreed with (13), they found that anti-CCP is a better diagnostic tool than RF. Anti-citrullinated peptide antibodies (ACCP) have a sensitivity of 40 to 80 %, a specificity of 81 to 100 %, and a good positive and negative predictive value for RA diagnosis.

Since the ACCP seropositivity observed in this study is almost similar to that reported from other studies, from Turkey (14). Netherlands (15). Moreover, the Data of this study agree with other studies (16). By showing a significant direct correlation between ACCP positivity and RA. This finding is especially important because, in addition to its use as a diagnostic marker, ACCP has also been identified as a predictor of poor prognosis in terms of disease severity and joint damage (17). This variation in anti-CCP seropositivity is due to that these antibodies are directed against different epitopes in citrulline-containing peptides and sera from the individual. patients may contain different subsets of ACCP antibodies (18).

Distribution of rheumatoid arthritis patients and control group according to interleukin-17 (Pg/ml)

The distribution of rheumatoid arthritis patients and as well as healthy control group was studied according to interleukin-17 (Table 3).

The results showed there was a significant association between the two study groups according to IL-17 level (P<0.016). As shown in table (4).

Table (4): Distribution of rheumatoid arthritis patients and control group according to interleukin-17

Subjects	Mean ± SE	T-test	P-Value
IL_17 Patients	148.061 ± 11.834	3.174**	0.016
IL_17 Control	121.126 ± 3.663		
* (P≤0.05), ** (P≤0.01).			

****T-test at significant 0.05.**

Interleukin-17 is one of the important cytokines that have a vital role in the development and progress of the pathogenicity of RA disease and is used as the target site for biological treatment. (19)**.** That interleukin 17 aid in the secretion of another pro-inflammatory cytokine (IL6, IL1, IL8,

and tumor necrosis factor-α) which can be manifested in synovial fluid and serum of RA patients (20,21). The important role of IL17 in some autoimmune diseases especially with RA disease and these findings may according to the fact that IL17 has the main role in stimulating other pro-inflammatory agents and aid in the accumulation of dendritic cells, monocytes, neutrophils, and TNFα that lead to inducing of inflammation than the progress of the disease to reach destruction of joint (22,23).

The present study showed a significant difference in IL17 between the RA patient group and the healthy control group and these observations were consistent with other previous studies in Iraq and other countries which showed. Serum IL17 concentrations were significantly higher in the Iraqi sample of RA patients compared to controls. This may help in the diagnosis of RA and suggest potentially an effective treatment (24). Another Iraqi study presents significantly higher levels of IL-17 among RA patients than the control group. This observation is consistent with several studies (25). The current study demonstrated significantly higher levels of IL-17 among RA patients than the control group, and this observation is consistent with a previous study (26)

Moreover, in the Tunisian population was found serum IL-17 concentration was significantly higher in RA patients compared to controls (27). This result comes agrees with the finding of Zhang, Li et al., (2011) (28). Who referred to the elevation of IL17 in RA patients in comparison with osteoarthritis patients and healthy controls. Some other studies reported an increase in IL 17 levels and its role in the pathogenesis of RA (29,30).

Genotype distribution and allele frequency of IL-17 rs763780 T\C in rheumatoid arthritis patients and control groups.

The genotype and allele frequencies of the IL-17 rs763780 T\C for the two study groups (controls and patients) are shown in table (5). All genotype frequencies of the control group and patients group confirmed the Hardy-Weinberg equilibrium (HWE).

Results from the table (5) show that the genotype and allele frequencies of IL-17 rs763780 T\C for the two study groups appeared that there were no significant differences between rheumatoid arthritis patients and the control group. Compared CC genotype between control and patients, the homozygous CC genotype was not associated with increased risk for rheumatoid arthritis CC (X^2= 0.438, OR =0.219); and the heterozygous TC genotype was no significant differences between patients and controls (X^2= 0.601, OR =0.593).

The data of allele frequencies of point mutation on IL-17 rs763780 T\C gene polymorphism in two study groups (control and patients) are presented in table (5). For the patients' group, the allele frequency of (T) was 75 %, but (C) allele frequency was 25 % according to the Hardy-Weinberg equation, while for control groups the allele frequency of (T) was 82 %, but (C) allele 18 % according to Hardy-Weinberg equation.

Table (5) shows that were no significant differences in allele frequencies of IL-17 rs763780 T\C gene polymorphism with the risk of rheumatoid arthritis in a sample of Iraqi patients.

Table (5): Genotype distribution and allele frequency of rs763780 T\C in patients and control groups

Genotype rs 763780 T/C	Patients Number (%)	Control Number (%)	Chi-Square (χ^2)	O.R. (C.I.)	P-value
TT	34 (68.00%)	39 (78.00%)	0.538 NS	Ref. =1	0.718
TC	7 (14.00%)	4 (8.00%)	0.601 NS	0.593	1.12
CC	9 (18.00%)	7 (14.00%)	0.438 NS	0.219	0.817
Total	50 (100%)	50 (100%)			
Allele	**Frequency**				
T	75	82			
C	25	18			
*** (P≤0.05), ** (P≤0.01), NS: Non- Significant.**					

OR: odds ratio; X²: Person's Chi Square.

Several studies have demonstrated the role of IL-17 in the pathogenesis of RA. The increased levels of IL-17 in the synovial fluid and tissue of patients with RA can induce inflammation and stimulation of osteoclast genesis through the up-regulation of the osteoclast differentiation factor (osteoprotegerin) (31). The ability of IL-17 to induce nitric oxide synthesis in cartilage, production of pro-inflammatory cytokines in peripheral blood macrophages, and collagenases in chondrocytes imply its role in cartilage biology (32).

These results agreed with the previous study (33). Which found that the gene polymorphisms of IL-17 rs763780 T\C in Polish patients with RA and healthy subjects were not correlated with susceptibility to RA in the Polish population. However, the IL-17 F variant was associated with parameters of disease activity, such as the number of tender joints, HAQ score, or DAS-28-CRP. And also agreed with Pawlik et al (2016). This suggests, that IL17F gene polymorphism is not the important factor associated with susceptibility and some clinical parameters of RA in a Polish population. Nevertheless, this hypothesis requires further investigation (34).

Moreover, eight studies evaluated IL-17F polymorphism, namely rs763780, (35-42). These were mainly case-control studies, except for the Bogunia-Kubik cohort study (38).

All eight publications assessed the association between susceptibility to RA and rs763780 polymorphisms – Bogunia-Kubik et al (2015). Reported a decreased risk of RA in patients with the TT genotype, and an overall protective effect associated with the T allele. (38) On the other hand, Marwa et al. (2017). Found that patients carrying at least one copy of the C allele were 6.4 times more susceptible to developing RA. (42)

The remaining studies found no significant associations between rs763780 polymorphisms and RA susceptibility (35,36,37,39,40), although there were reports on those polymorphisms being significantly associated with tender joints, disease duration, higher creatinine levels and lower sedimentation rate (37,40,42).

In our study IL17F gene polymorphism was not the factor associated with susceptibility to RA, moreover, there were no statistically significant associations between these polymorphisms and age of disease diagnosis, rheumatoid factor, joint erosions, and extra-articular manifestations.

Concerning IL-17F, this cytokine contributes to neutrophil recruitment and activation through the stimulation

of cytokine and chemokine production. Several studies evaluated the effect of rs763780 on RA susceptibility, which has also been shown to be associated with other inflammatory diseases (43,44,45). The mutated variant of this polymorphism is responsible for a His-to-Arg substitution at amino acid 161, which can change the conformation or molecular expression of IL-17F (46).

In fact, in vitro experiments demonstrated that, unlike wild-type IL-17F (TT genotype), this mutated variant could not activate the mitogen-activated protein kinase pathway, with the CC genotype associating with lower cytokine production and chemokine production in bronchial epithelial cells. Our meta-analysis showed that the TT genotype was significantly more frequent in healthy individuals than in RA patients, while the opposite was observed for the CT genotype. Previous meta-analyses also found (47) that carriers of the rs763780 C allele, CT, or CC genotypes had higher risks of developing RA than subjects with the T allele or TT genotype.

As we talked about above in the genetic analysis of the gene IL-17F rs763780 and we said that There are some potential limitations to our study, which could contribute to false positive or negative results. First, our sample size may not be large enough to detect an association of a gene with the same effect of RA. Our control groups were smaller than RA groups, so the power of this study is not too high. Nevertheless, the analysis of polymorphisms should rely on the clinically well-described group and not just on the sample size. Unfortunately, in our study, only one SNP was tested in patients with RA and control. These findings demonstrated that the IL-17 F variant might be associated with increased disease activity in Iraqi patients with RA.

Serum IL-17 level and its association with the IL-17 of rs763780 T\C genotypes:

The serum IL-17 level and its association with the IL-17 rs763780T\C genotypes between the studied groups (patients and control) were calculated as illustrated in the table (5). There was a significant increase of IL-17 in the serum of the patients with RA compared with the control group. However, when the comparison within these genotypes according to IL-17, serum levels showed high significance with a P value ≤0. 0.0001 **0.0001 **0.0001 ** respectively in both patient and control.

Table (6): Serum IL-17 level and its association with the IL-17 F rs763780 T\C genotypes

Genotype 763780 T/C	IL-17		P-value
	Patients	Control	
TT	195.32 ±17.65	60.96 ±5.17	0.0001 **
TC	160.89 ±16.54	66.71 ±13.11	0.0001 **
CC	323.34 ±123.28	74.71 ±10.06	0.0001 **
LSD value	173.43 NS	32.67 NS	---
This means having different letters in the same column differed significantly. * (P≤0.05), ** (P≤0.01).			

Inflammatory processes and cytokines are important in the etiology of RA. Individual differences in cytokine levels are a probable explanation for disparities in illness susceptibility and severity and are mostly due to (SNPs) in the genes that code for cytokines (6). Interleukin-

17 is a pro-inflammatory cytokine made by the memory CD4 + T cells after activation and is involved in amplifying inflammatory response by recruiting immune cells such as neutrophils and monocytes and inducing other pro-inflammatory molecules (48).

Some studies show a variety of IL-17F roles in the pathogenesis of airway inflammation due to an allergic reaction and may be associated with the activation of some T lymphocytes in the recruitment and activation of neutrophils in the airway (26). Among the IL-17 cytokine family members, IL-17F shows the highest amino acid sequence homology (50%) to IL-17A, while only 10–30% sequence identity is seen between IL-17A and the other family members (49). In another meta-analysis for IL-17 levels in osteoarthritis (OA) present, The OA patients showed significantly higher IL-17 levels than the control subjects (50).

Interleukin-17 is a pleiotropic cytokine that participates in tissue inflammation and destruction by inducing the expression of proinflammatory cytokines and matrix metalloproteases. Agarwal et al, (51). An enhanced expression of IL-17 has been observed in the rheumatoid synovium and synovial fluids of patients with early RA (52). Moreover, IL-17 has become a new therapeutic target for animal models with collagen-induced arthritis and human RA (53,54).

This study agreed with the previous study in Iraq which found the serum IL17 concentration was significantly higher in the Iraqi sample of RA patients compared to controls and this may help in the diagnosis of RA and suggest a potentially effective treatment.

Similar findings were reported by other studies. Chen et al (55) investigated the effects of tumor necrosis factor (TNF)-a inhibitors on circulating Thelper-type17 (Th17) cellsandTh17 related cytokines (RA) in 48 RA patients both before(baseline) and six months after anti-TNF-a therapy and found significantly higher baseline frequencies of serum IL17 and IL6.

Moreover, Recent studies have reported that IL17 has an important role in RA pathogenesis. Hueber et al (53) and combined inhibition of IL17A and -F can control RA inflammation and joint destruction. Genovese et al. (54). In addition to a significantly increased level in RA synovial tissue and readily detected in RA SFs (Shahrara et al,2008).

Roso *et al*. (56) assessed IL-17 patterns in the synovium, serum, and synovial fluid from 30 treatment-naïve, early rheumatoid arthritis patients and compared it to 29 control osteoarthritis patients and found in early RA patients, strong correlations of serum and SF IL-17A levels compared to controls. Liu et al (57) evaluated the role of interleukin IL-17 in anxiety and depression of 18 patients with rheumatoid arthritis compared to 18 healthy controls and showed that serum IL-6 and IL-17 levels were significantly higher in RA patients than those of healthy subjects.

Interestingly, we found a direct strong significant correlation between serum IL17 and IL6 concentrations in RA patients. This may suggest that controlling IL-6 activities is potentially an effective approach in the treatment of RA that can reduce serum IL17 and subsequently improve patients with RA. (58) A study by Kimura and Kishimoto (2010) reviewed the role of IL-6 in regulating Th17/Treg balance and described the critical functions of

IL-6 and Th17 in immunity and immune pathology.

The main limitations of the present study were the small size of the studied sample and the short period of the study and these can be solved by larger prospective studies with longer periods of follow-up to support the reported data. Yet, despite that, this study has points of strength like strict inclusion and exclusion criteria, and defined data measurement and collection.

Conclusion

The ACCPA level and IL-17 level results revealed a significant difference ($P<0.01$) association between the two study groups in ACCPA level, the mean ± SE serum levels of ACCPA in rheumatoid arthritis patients were significantly higher as compared to healthy control (10.67 ±0.901 versus 6.70 ±0.390 U/ml), while in IL-17 level, the mean ± SE serum levels of IL-17 in rheumatoid arthritis patients were significantly higher as compared to apparently healthy control (148.06 ±11.834 versus 121.12 ±3.663).

The genotyping and allele frequencies of IL-17 rs763780 T/C for the two groups appeared that there were found to be negative significant relation in genotype between patients and controls.

Compared TC genotype between control and patients, the heterozygous TC genotype was significantly different from controls ($X^2=0.601$, OR=0.593); and the CC genotype was no associated significant risk for RA ($X^2=0.438$, OR=219).

In addition, allele frequency for the C allele were no significant differences in allele frequencies of IL-17 rs763780 T\C gene polymorphism with the risk of RA in a sample of Iraqi populations.

Moreover, the association between the serum IL-17 level and 17 rs763780 T\C genotypes, in patients with RA significantly increased ($P<0.05$) compared to controls.

The conclusion obtained from this study was that serum ACCPA determination was of important value for the diagnosis of rheumatoid arthritis, and an increase in IL-17 plays an important role in the development risk of RA.

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