Iraqi Journal of Biotechnology, 2022, Vol. 21, No. 2, 46-60

Study the Association of *IRAK1* Gene Polymorphism and Some Immunological Markers with the Risk of Rheumatoid Arthritis Incidence in Sample of Iraqi Patients

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Received: 1/6/2022 Accepted: 21/8/2022 Published: December 20, 2022

Abstract: Rheumatoid arthritis (RA) is a multifactorial autoimmune disease affecting 1–2% of the population worldwide. It is more common in women than in men, predominately aged 40-60 years. Interleukin 1 receptor associated kinase 1 (IRAK1) gene related with the susceptibility to autoimmune diseases and might be one of the important risk factors of RA. The objective of this study is to identify the association of single nucleotide polymorphism SNP of IRAK1 (rs3027898 C>A) gene with Rheumatoid arthritis susceptibility in a sample of Iraqi patients and to examine whether this polymorphism can influence the severity and activity of the disease. study consists of two groups: sixty RA patients (53 females and 7 males, mean age 46.35 ±1.43) were diagnosed with rheumatoid arthritis clinically and conformed by rheumatologist examination and laboratory tests according to the 1987 American College of Rheumatology criteria for RA revised principles for the classification of RA while other sixty healthy individuals (36 females and 24 males. mean age 34.15 ±1.54) set as control group. Polymorphisms were genotyped by using High Resolution Method (HRM) real time PCR for genotyping assay. The mean age of RA patients was (46.35 ±1.43) years, females' ratio was higher than the males. No significant effect of family history seen between the healthy and RA patient groups. The observed genotype frequencies for the polymorphism *IRAK1* (rs3027898 C>A) in RA patients and control subjects show no Significant differences in the frequency of *IRAK1* rs3027898 alleles (CC, CA, AA) and genotypes were observed between RA patients and controls P-value (0.317, 0.594, and 0.071) respectively. The C allele frequency values were (0.52 and 0.45) for apparently healthy subjects and RA patients, respectively, the values of allele frequency of the A allele were (0.48 and 0.55) of apparently healthy with RA patients, respectively. The CC percentage in RA patients and healthy controls (35%, 30%), the CA percentage (33.33%, 30%) while AA percentage (31.67%, 40%) respectively. In conclusions, the three genotypes (CC, CA, AA) of the Interleukin 1 receptor associated kinase 1(*IRAK1*) Gene in the (rs3027898 SNP) all seem do not have risk factor for RA in the studied samples of Iraqi population.

Keywords: *IRAK1* Gene, rs3027898, HRM.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune, chronic, multisystem and inflammatory disease that affects 1-2% of the population that is distributed worldwide, regardless of race, sex, nationality, age, ethnicity, etc. (1). It is more common in women than in men

by about threefold to fivefold, predominately at age 40-60 years (2). The clinical manifestation involves chronic inflammation of the synovial joints, hyperplasia that is a result of the overgrowth of synoviocytes, and bone erosion (3) as well as joint cartilage degradation leads to significant functional disability that causes stiffness, especially in the morning, pain, and loss of mobility in the joints (4). The epidemiology of RA is geographically varied and this variation is a result of many factors such as geographic resident region, socioeconomic standing, and exposure to genetic and environmental influences. In the North of Africa for example in Egypt the prevalence of RA is about 0.09% while in Libya elevates to 0.15% meanwhile the occurrence of RA in both in Afghanistan and Iran the distribution is almost equal which is about 0.15% not to mention the gulf countries the prevalence shows in Bahrain 0.2% while decreasing in Kuwait in about 0.07% (5). In Iraq in particular, specifically in Babylon province, the incidence of RA was observed during the period 2001-2011 at about 3.02% (6). Despite of rheumatoid arthritis has unknown etiology, the genetic factor thought to play an important role in the RA pathogenicity, in fact approximately 50% of people have a genetic susceptibility to RA (7). The HLA-DRB1 gene is the main hereditary factor for RA, but the HLA genes account for 33.3% of the disease's hereditary risk (8). Common amino acid sequences in the peptide-binding groove are shared by disease-associated alleles (the so-called shared epitope) which is mainly correlated with the seropositivty of autoantibodies against citrullinated peptides (ACPAs) and autoantibodies against IgG (rheumatoid factor) (9).There are other non-HLA genes that have been linked to RA susceptibility (10). Interleukin 1 receptor associated kinase 1 is the first member of the four IRAK family members, which consists of *IRAK1*, *IRAK2*, *IRAK3* (also known as *IRAK-M*) and *IRAK4*. An N-terminal death domain (DD) and a kinase domain are all found in IRAK members (11). In addition to the (DD) and kinase domains, *IRAK1* has a proline/serine/threonine-rich domain (ProST) and a C-terminal domain. In order to interact with other IRAK family members or proteins, *IRAK1* uses its proST domain, which is made up of serine, proline, and threonine amino acid residues. In addition to the (DD) and kinase domains, *IRAK1* has a ProST domain and a C-terminal domain. In order to interact with other IRAK family members or proteins, *IRAK1* uses its proST domain, which is made up of serine, proline, and threonine amino acid residues. Furthermore, *IRAK1* has an invariant lysine including its central kinase domain. The invariant lysine functions as a mediator for catalytic function and kinase activity as well as an ATP binding site (12). *IRAK1* gene is located on chromosome X (Xq28) including 14 exons besides that it binds to intracellular domain of IL1R contains many single nucleotide polymorphisms which induce the signaling pathways. The *IRAK1* gene related with the susceptibility to autoimmune diseases and might be one of the important risk factors of RA (13). The Toll-like receptor (TLR) subfamily and the interleukin-1 receptor (IL-1R) subfamily make up the TIR family, which has a unique sign called the TIR domain which the pathogen-mediated inflammation is known to contain

members of the TIR family (14). *IRAK1* can recognizing a variety of ligands and taking part in the inflammation and is accumulated upon TLR stimulation recent research suggests that activating the innate immune system, specifically elevated *IRAK1* expression and activity, may enhance the production of inflammatory proteins like C-reactive protein (CRP) which results in the activation of the signal transducer and activator of transcription 3 (STAT3) factor and transcription of the CRP gen (15). A wide and unique signaling module, comprising MyD88 and the *IRAK* family, is activated by the TIR domain, the structure for a succession of protein-protein interactions (16). Nuclear factor NF-κB is activated by this binding, allowing the signal to enter the nucleus and carry out its intended function, the inflammatory conditions are reduced as a result of *IRAK1* inhibitors that suppress of NF-κB activation (17). The activation of nuclear NF-κB is the most remarkable event of the inflammatory response is that it significantly activates the innate and adaptive immune system's inflammatory response (14). Mature miRNA-146a can bind to many target mRNAs, including interleukin-1 receptor-associated kinase 1 (*IRAK1*) that has a number of SNPs that influence its gene expression and functions (18). In this article we will explore the association of the gene *IRAK1* (rs3027898SNP) and rheumatoid arthritis in the Iraqi population.

Subjects, materials and methods

Study consist of two groups, sixty patients with the rheumatic arthritic disease (53 females and 7 males) and sixty as apparently healthy subjects (control) and personal information such as: age, weight, height, medical family history, the samples were admitting Baghdad Teaching Hospital-Medical City for chronic arthritis diseases department in Baghdad, during the period from December 2021 and February 2022. The study design was approved by the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad. Writing informed consents were obtained from all patients and apparently healthy control group; all patients were diagnosed according to clinical examination by a rheumatologist examination and selected according to the criteria of the 1987 American College of Rheumatology criteria for RA revised principles for the classification of RA.

Genomic DNA extraction

Venous blood samples were collected of both healthy and RA patients, DNA was extracted from whole blood using (Easy Pure® Genomic DNA Kit, TransGen biotech, China). DNA concentration and purity was estimated by Nanodrop-2000 spectrophotometer (NanoDrop™ 2000/2000c Spectrophotometers, USA) and the purity ranged between 1.8 and 2.0, DNA bands were visualized using UV light after electrophoresis in a 1% (w/v) agarose gel. Genotyping was performed using high resolution melting (HRM) real-time PCR curve analysis detecting systems (Qiagen Rotor gene Q-Real-time PCR system, Germany). Extracted genomic DNA was amplified in a 20 µl solution containing (10 µl of master mix, 1 µl of Forward Primer and Reverse Primer respectively, 3 µl of DNA template and completed with 5 µl of D.W).

PCR Primers for *IRAK1* Gene

Single nucleotide polymorphisms (SNPs) of *IRAK1* rs3027898 was analyzed in the extracted DNA by using specific primers and HRM PCR super Mix (TransStart Tip Green qPCR Super Mix, TransGen biotech, China) The following PCR primers were used for *IRAK1* rs3027898: 5'AGCCCAGAGTAGTAACAAGACC 3' (Forward) and 5' GTTGCACCGAAAGCCTTGAG3' (Reverse), shown in Table (1).

Table (1): Primer sequences.

Gene	Primer	Sequence	Product Size	Tm
IRAK1 rs3027898	Forward	5'AGCCCAGAGTAGTAACAAGACC3'	75	60°C
	Reverse	5' GTTGCACCGAAAGCCTTGAG3'	83	66°C

Primers that used in this study were designed according to their reference sequence in the database of National Center for Biotechnology Information (NCBI). Extracted genomic DNA was amplified in a 20 µl solution containing (10 µl of master mix, 1 µl of Forward Primer and Reverse Primer respectively, 3 µl of DNA template and completed with 5 µl of D.W), Table (2).

Table (2): The reaction components of PCR.

Component	Volume (µl)
TransStart Tip master mix	10
Forward Primer	1
Reverse Primer	1
DNA Template	3
D.W	5
Total volume	20

PCR Program

Quantitative Real time PCR (qRT-PCR) was carried out by using the (Qiagen Rotor gene Q-Real-time PCR system, Germany). The *IRAK1* polymorphism levels fold change was quantified by measuring the threshold cycle (Ct) employing the HRM PCR Master Mix kit component. The kit is optimized to even enable successful analysis of genomic loci that are difficult to amplify also highly suitable for mutation scanning and can be used to screen samples for unknown mutations, not to mention analysis of the genetic variations (SNPs, mutations, methylations) in PCR amplicons. HRM characterizes double-stranded PCR products based on their dissociation (melting) behavior as they transition from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) with increasing temperature. PCR products can be discriminated according to sequence, length, GC content, or strand complementarity, down to single base pair changes. Previously unknown and even complex sequence variations as seen in challenging genotyping applications can be easily analyzed. (Table 3) shows the HRP thermal profile.

Table (3): Thermal profile of HRM

Step	Temperature (ºC)	Duration	Cycles
Enzyme activation	94	1 min	1
Denaturation	94	5 sec	40
Annealing	58	15 sec	
Extension	72	20 sec	1
HRM	65-90	0.2 sec for 1 degree	1

Determination of anti-CCP and ANA titer using enzyme-linked immunosorbent assay kit

The Principle of both immunological markers (Anti-CCP and NAN) include the qualitative enzyme immunoassay method that used in this assay. The CCP/ANA antigen has been pre-coated on the microtiter plate included in the package. Anti-human IgG conjugated Horseradish peroxidase (HRP) is pipetted into the wells, and any antibodies specific for CCP/ANA present will attach to the pre-coated antigen to produce a blue color product that changed into yellow after adding acidic stop solution. The color intensity is measured. The density of yellow is proportional to the Anti-CCP/ANA amount of sample captured in plate. The O.D. is read at 450nm in a microplate reader, and then the concentration of Anti-CCP/ANA can be calculated.

Determination of rheumatic factor and C-reactive protein seropositivity in latex test

This kit based on the agglutination of the serum. In case the rheumatoid factor (RF) is present in the serum, a visible agglutination seen on the slide of suspension of latex particles that coated with human gamma-globulin1. In case C-reactive protein (CRP) is present in serum at a concentration of six milligram per liter, causes a visible agglutination on the slide of a suspension of latex particles coated with anti-CRP.

Erythrocyte sedimentation rate (ESR)

Westergren method is commonly used for ESR application. the procedure involves mixing 1.6 ml of venous blood with 0.4ml of 3.8% trisodiumcitrate dihydrate solution in a vacutainer tube then Westergren pipette is filled up to zero by inserting it into the vacutainer tube afterwards placing it on ESR rack and setting the timer for one hour After one hour the level of erythrocyte sedimentation is measured in millimeters (mm).

Statistical analysis

The Statistical Analysis System-SAS (2018) program was used to detect the effect of difference factors in study parameters. T-test and Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage 0.05 and 0.01 probability in this study.

Result and discussion

Distribution of rheumatoid arthritis patients and control group according to gender

In the comparison between RA patients and healthy control groups were estimated according to gender, it shows a significant high difference. The percentage of RA was in women highly increased (P-value = 0.0066) with (88.33%) as compared with men with (11.67%) as shown in (figure 1). This

result agrees with other Iraqi studies (6) and (19) who found the percentage of RA incidence in females are more than in males, while in other Iraqi study done in Duhok city show the percentage of men is more significant than in woman (20). Nilsson *et al*. (2021) suggested in their study that sex hormones are believed to be involved in the development of RA, about (67%) of women were seropositive (21). As estrogens, androgens, and prolactin can influence the immunological response by using androgen and estrogen receptors (ER), both receptors are expressed by progenitors and mature cells, suggesting that sex hormones might affect immune cell development directly. Estrogens have a distinct modulatory impact under normal circumstances and during autoimmune disease, where they have a biphasic effect, in which lower levels stimulate the immunological response, whereas higher levels inhibit it (22).

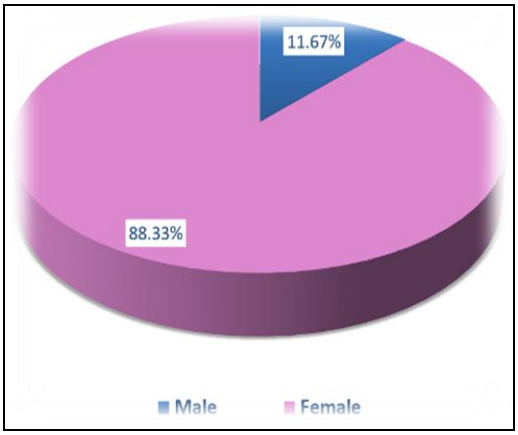

Figure (1): RA patients distribution according to gender.

Distribution of rheumatoid arthritis patients and control group according to family history

The family history of RA disease was obtained from both healthy individuals and RA patients. In this study no significant effect seems of family history on RA patients compared to healthy individuals, in which (P-value = 0.107) for both groups. The percentage of RA distribution in family history in RA patients was (21.67%), while in healthy individuals the percentage dropped to (13.33%). These results disagrees with a study done by (23) that concluded the first-degree relatives of RA patients have a 5-fold higher chance of developing RA than people in general. Sixty percent of the phenotypic variance in RA was caused by shared environmental variables and genetic factors combined, and genetic transmission accounted for three quarters of this familial transmission. This disagreement suggests that a higher study of population of RA patients may effects on the results.

Distribution of rheumatoid arthritis patients and control group according to BMI

Patients' body mass index (BMI) is calculated to determine whether they are a healthy weight or obese. Weight is compared to height to calculate BMI category, with BMI of (18.5 to 24.9 Kg/ m^2) indicating normal weight, BMI of (25 to 29.9 Kg/ m^2) indicating

overweight, and BMI of (30 Kg/ m^2) indicating obese. In this study, the observations of the BMI Kg/m^2 mean show no significant differences between RA patients and healthy individuals. Both seem to be overweight, but a significant variation between the mean of BMI Kg/m^2 and mean age, in which (P-value = 0.0004) of RA patients' BMI and (P-value = 0.0001) of age, the BMI Kg/m^2 mean of RA patients (29.48 ±0.64) and mean age (46.35 ±1.43) years old. Age-related changes in fat distribution result in an increase in visceral fat, which is more significant in women than in men. Fat is also being deposited more and more in the liver and skeletal muscle. The key factor causing poor glucose tolerance in the elderly is greater visceral fat, through locally released free fatty acids, increased intramuscular and intrahepatic fat contributes to decreased insulin action, a role is also played by increased pancreatic fat and deteriorating b-cell activity (24).

Distribution of rheumatoid arthritis patients according to family history

All of the sixty RA patients had their medical information checked by their medical reports and physician to access the other significant comorbidities in RA patients. While all of the sixty healthy controls did not suffer any confirmed medical problems by a physician, the following findings were shown respectively: (Urinary Tract Infections UTI and Hypertension showed the same percentage of 11.67% (P-value = 0.0498); (Hyperglycemia 6.67%, P-value= 0.092); and (Osteoporosis 3.33%, P-value 0.0052), (figure 2). In this study no significant effect seems of family history on RA patients compared to healthy individuals. These results disagrees with a study done by Kuo *et al.* (23) that concluded the first-degree relatives of RA patients have a 5-fold higher chance of developing RA than people in general. Sixty percent of the phenotypic variance in RA was caused by shared environmental variables and genetic factors combined, and genetic transmission accounted for three quarters of this familial transmission. This disagreement suggests that a higher study of population of RA patients may effects on the results.

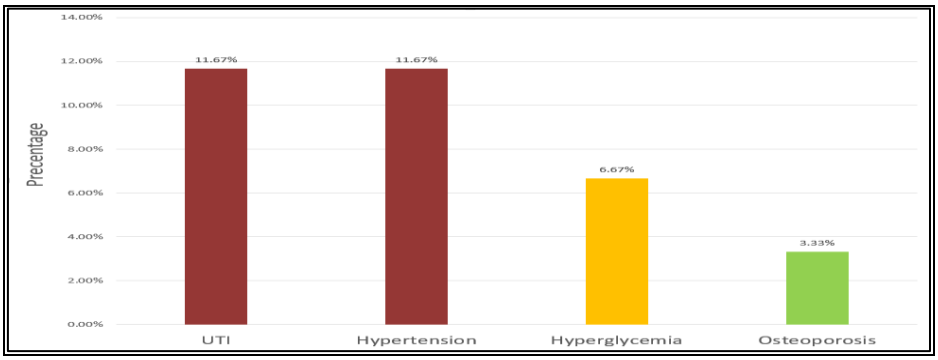

Figure (2): Other significant RA comorbidities.

Distribution of rheumatoid arthritis patients according to erythrocyte sedimentation rate (ESR)

The results of ESR levels in this study show an increase in the patient group (newly diagnosed and treated)

compared to the control group, an increased significant difference (p-value = 0.0001) in which the mean ± SE of ESR in patients is (48.06 ± 3.92 mm/hour) while it decreases in healthy individuals (20.90 ± 2.33 mm/hour). In the present study, these findings of ESR indicates the existence of an immune system-induced inflammatory response to RA. The results are similar to other Iraqi researchers (25) (26).

Real time PCR quantification of *IRAK1*

The observed genotype frequencies for the polymorphism in the 3'-UTR downstream of *IRAK1* (rs3027898 C>A) in RA patients and control subjects show no Significant differences in the frequency of *IRAK1* rs3027898 alleles and genotypes were observed between RA patients and controls, table 4, P-value (0.317, 0.594, and 0.071) respectively. The C allele frequency values were (0.52 and 0.45) for apparently healthy subjects and RA patients, respectively. The values of allele frequency of the A allele were (0.48 and 0.55) of apparently healthy with RA patients, respectively. The PCR output results are shown in figure 3. Concerning rs3027898 SNP of *IRAK1* gene, the results of the present study disagree with Shaker *et al*. (27) and Ayeldeen *et al*. (28) who studied on rs3027898 of *IRAK1*gene in Egyptian population, they found that a statistically significant difference in the frequency of the A allele and the genotype distribution for CA was found at *IRAK1* rs3027898 (P=0.01, P=0.001). Whereas, RA patients were more likely to carry the A allele and the heterozygous genotype CA than were controls. *IRAK1* rs3027898 has been also studied in Greek population, the genotyping of Greek patients with RA has found a link between *IRAK1* rs3027898 and RA (18). While a research conducted in Tunisia (29) their study's major finding was that there was no correlation between RA and *IRAK1* rs3027898.

Table (4): Comparison of the genotype and allele frequencies of *IRAK1* gene polymorphism (rs3027898 C>A) between patients group and control group.

Genotype IRAK1 rs3027898	Control No. (%)	Patients No. (%)	Chi-Square (χ^2)	P-value	O.R. (C.I.)
CC	21 (35.00%)	18 (30.00%)	1.052 NS	0.317	1: Reference
CA	20 (33.33%)	18 (30.00%)	0.882 NS	0.594	0.422 (0.27-0.82)
AA	19 (31.67%)	24 (40.00%)	2.74 NS	0.071	0.297 (0.08-0.552)
Total	60 (100%)	60 (100%)			
Allele	Frequency				
C	0.52	0.45	0.419 NS		
A	0.48	0.55			
NS: Non-Significant.					

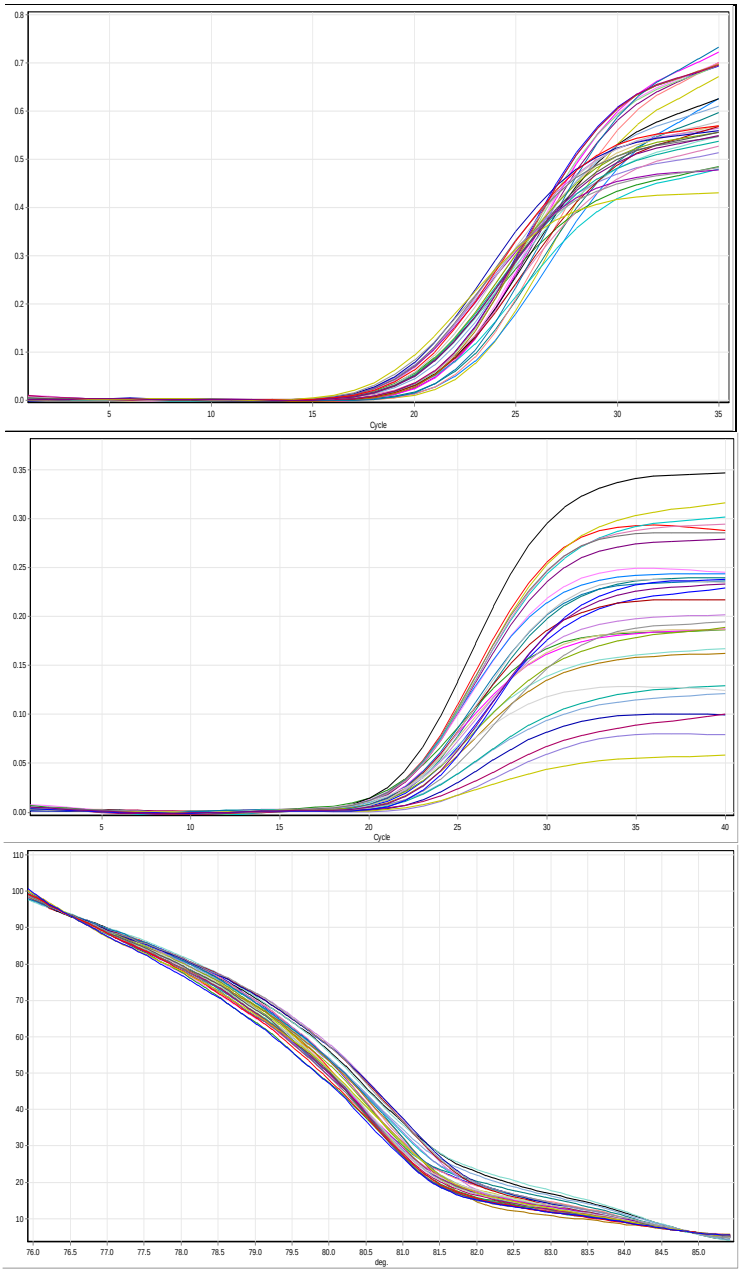

Figure (3): The results output of HRM in RT-PCR for the three genotypes in rs3027898 of *IRAK1* gene.

Since the *IRAK1* gene is encoded on the X chromosome, this study has evaluated how different RA risk according to gender is impacted by *IRAK1* rs3027898 genotypes on both males and females between patients and apparently healthy controls, as shown (table 5). There are no significant differences in AC and AA allele's genotype percentage between apparently healthy subjects and RA patients as related with rs3027898 at *IRAK1* gene, although the genotype CC seems to be more significant in healthy controls than in patients, the three alleles (CC, AC and AA) show no evident of the RA risk relation to gender. The obtained results are different to what Shaker *et al*. (2018) that have concluded, which when

compared to men, females showed a significantly higher risk of RA associated with the *IRAK1* rs3027898 CA genotype (P=0.001) and A allele (P=0.001). Regardless to age and gender the differences in ethnicity and sample sizes may be the cause of discrepancies seen in this study, the findings of the largest studies conducted in Caucasian and Asian populations indicate that *IRAK1* region appears to be more significantly related with RA in the Asian population than in the Caucasian population (30). Another hypothesis is that *IRAK1* rs3027898 is in linkage disequilibrium with the true disease-associated allele in certain groups but not others, and it is known that linkage disequilibrium differs by race (29).

Table (5): Comparison of the genotype and allele frequencies of *IRAK1* gene polymorphism (rs3027898 C>A) between patients group and control group according to gender.

Gender	Genotype	Control No. (%)	Patients No. (%)	P-value
Male (No=31)	CC	8 (25.81%)	1 (3.23%)	0.0063 **
	AC	8 (25.81%)	3 (9.68%)	0.071 NS
	AA	6 (19.35%)	3 (9.68%)	0.114 NS
Female (No=89)	CC	15 (16.85%)	19 (21.35%)	0.602 NS
	AC	15 (16.85%)	19 (21.35%)	0.602 NS
	AA	6 (6.74%)	15 (16.85%)	0.109 NS
** (P≤0.01), NS: Non-Significant.				

Serological study

Distribution of rheumatoid arthritis patients according to RF

All sixty RA patients tested positive for RF (100%) while only 10% of the sixty control subjects tested positive for RF as shown in figure 4. The results of RF test show all patients were seropositive for RF, while in other study done on a Kurdish population in north of Iraq (31) in which (67.69%) of RA patients were seropositive for RF. However, it is uncertain if people in the general population who do not have rheumatoid arthritis have increased levels of rheumatoid factor is linked to the development of rheumatoid arthritis later in life (32).

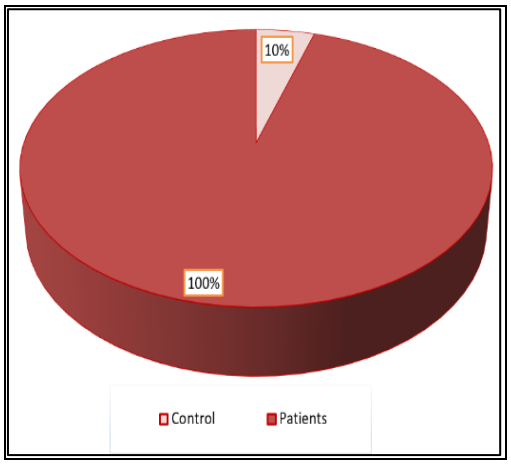

Figure (4): Distribution of RF results in RA patients and healthy controls.

Distribution of rheumatoid arthritis patients according to C-reactive protein (CRP)

The increased percentage of CRP seropositive tests is mostly seen in the sixty RA patients (60%), whereas only 10% of the sixty controls show seropositive CRP as shown in figure 5. In this study involved CRP test, in rheumatoid arthritis, C-reactive protein (CRP) is often measured as a marker of systemic inflammation (RA) although it is not specific test to RA. It functions as an immunological regulator, which is crucial in the inflammatory pathways linked to RA and stimulates atherogenic effects. CRP is also linked with systemic inflammatory comorbidities are prevalent in RA, and CRP has been related to an increased risk of depression, diabetes, metabolic syndrome, lung disorders, and cardiovascular and metabolic diseases (33).

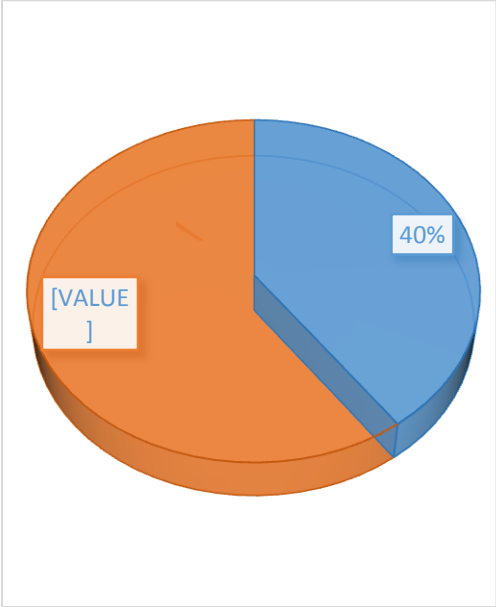

Figure (5): Distribution of CRP results in RA patients and healthy controls.

Evaluation the concentrations of anti-cyclic citrullinated peptide (Anti-CCP) by ELISA

The serum level of the Anti-CCP test, which was done by Elisa, gave results that revealed a highly significant variation in the mean SE of Anti-CCP between RA patients and healthy controls (P-value = 0.0001). The mean of Anti-CCP in controls was 0.244 ± 0.004, while in RA patients it was 0.504 ± 0.006 as shown in figure 6. The seropositivity of Anti-CCP test in this study demonstrated by Eker *et.al* (34) that most of RA patients have elevated serum level of Anti-CCP.

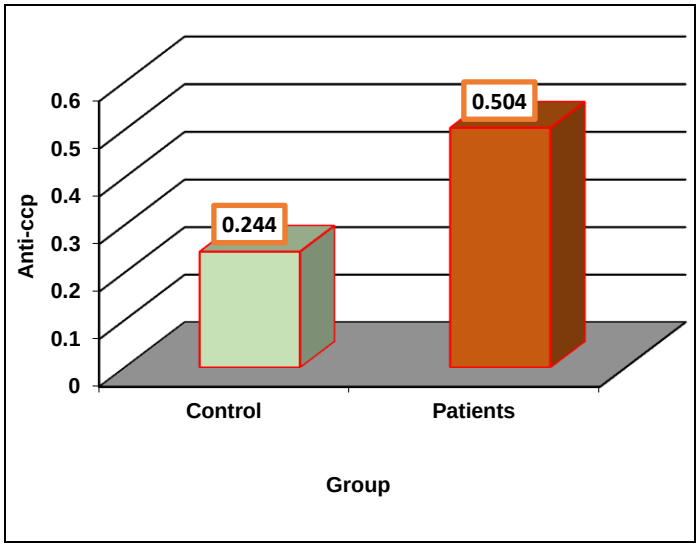

Figure (6): Comparison between control and patients groups in Anti-CCP.

Evaluation the concentrations of anti-nuclear antibodies by ELISA

The serum levels of (ANA), (figure 7) show a highly significant increase among RA patients compared to the control group, in which the mean ± SE of ANA in patients (0.528 ±0.025) while in controls (0.210 ±0.003) and the P-value (0.0001). The ANA test involved in this study show increased significant of ANA serum level which agree to Paknikar *et al.*, (35) that revealed a 25% of patients were seropositive , while in an Egyptian study (27) and (36) show low mean ± SE of ANA positivity.

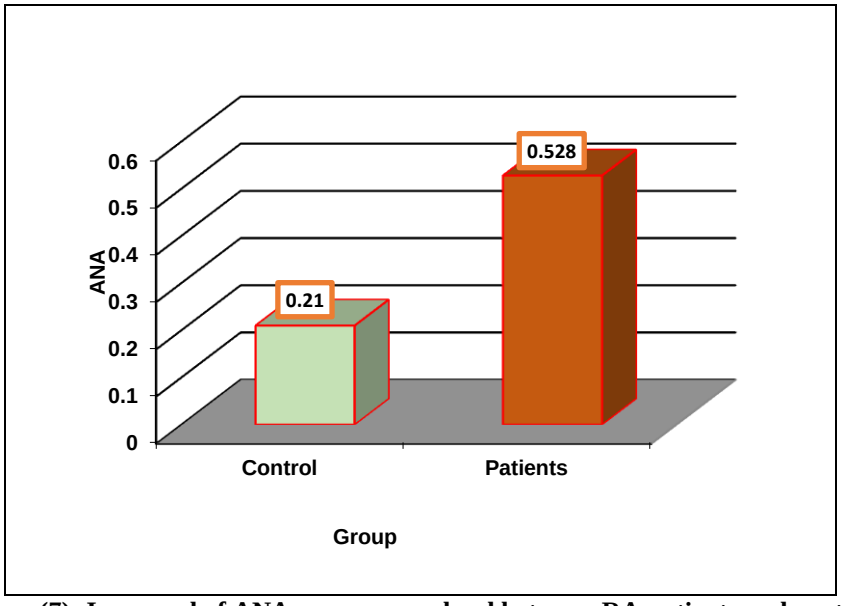

Figure (7): Increased of ANA mean serum level between RA patients and control.

Conclusion

The three genotypes (CC, CA, AA) of the Interleukin 1 receptor associated kinase 1(*IRAK1*) Gene in the (rs3027898 SNP) all seem do not have risk factor for RA in the studied samples of Iraqi patients.

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