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MOLECULAR STUDY OF POLYMORPHISM FOR GENE TNF-ALPHA USING ARMS-PCR TECHNIQUE FOR PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT : Rheumatoid Arthritis is one of the most common diseases of the present, which develops rapidly and affects both sexes with different ages. Since there is no study that deals with the relationship between the formal polymorphism of a tumor necrosis factor at the site (G-308A) of the Country level. This study was designed to investigate the association between the polymorphism of the alpha tumor necrosis factor at the site (G-308A). And its relationship with rheumatoid arthritis disease in a group of Iraqi patients infected with this disease using the technique of Tetra-Primer ARMS-PCR the 100 blood samples were collected from patients with rheumatoid arthritis of both sexes, who were diagnosed by specialists from different areas of Salah Al-Din governorate and 50 blood samples from healthy people as a control group without any disease and at roughly similar ages, basic laboratory tests (RF, ESR, CRP). For the purpose of confirming the injury of patients and non-infected healthy people disease Rheumatoid Arthritis, where DNA was extracted from all samples and performed molecular diagnosis of mutation (G-308A) using the technique of Tetra-Primer ARMS-PCR. The multiplication results were then migrated on the agarose gel , according to the results of this study, all patients with arthritis have a high rate of ESR and CRP. Most have a positive rheumatoid factor test compared with the healthy group. In addition, the majority of the patients were female. On the other hand, the results showed that the allele frequency G was found in 52% patients and 98% control group, while the allele frequency A was 48% in the patients group and 2% in the healthy group.

Key words : Rheumatoid arthritis, gene TNF-Alpha, polymorphism.

INTRODUCTION

Arthritis is one of the most common diseases at present. It takes many forms and develops rapidly and affects both sexes of different ages and there are different types including osteoporosis, rheumatoid arthritis, severe sepsis, gout, rheumatoid arthritis, psoriatic arthritis, stell disease and juvenile arthritis. The severity of the disease varies according to the stage of the disease , In general rheumatoid arthritis is the most common type of arthritis (Muhsen and Aldujaly, 2011).

Rheumatoid arthritis disease is described by Thomansydenhame and lavdry in 1800. But the first to be called rheumatoid arthritis is 1859 (Mainiand Feldman, 1998).

Rheumatoid arthritis is an autoimmune disease. It is a chronic systemic inflammatory disease that affects the synovial joints mainly and leads to erosion, deformity, joint destruction and loss of function (Gupta *et al*, 2005; Haslett *et al*, 2002; Klippel *et al*, 2001; Robbins *et al*, 2001). The experts divided the disease into two types. The first is Oligoarthritis (which is the least common) and remains for a few months and the cure occurs without permanent disability. The second type is polyarthritis (the chronic type), which lasts several years and may last a lifetime (Klippel, 2001; Mercy Medical Center, 2005; University of Maryland Medical Center, 2004).

Rheumatoid arthritis is present in different places of the body, including the jaw, knees and arm. It mainly affects the synovial joints and leads to erosion, deformity, destruction of the joint and loss of function It is often (Muhsen and Aldujaly, 2011) very widespread in the world. It is a common disease in many countries and affects women more than men in the rate of 3:1 (Hyde, 2000). While his percentage was in Iraq 2.9:1 (Abass, 2003) as well (Alqasiy, 2003) that ratio 9.3:1 is the infection of women to men, where the disease in Iraq is now one of the most dangerous diseases and the most affected among other diseases and found that the prevalence of nearly 1%, similar to the rate of spread in other countries Rheumatoid arthritis occurs during adolescence and increases moderately in the third decade with a severe increase in the fourth decade of life (Winchester, 1995).

The incidence of infection between monozygote twins is 30% more than twins are Heterozygote is 5% (Vander Heijde, 1992). As well as among relatives of the first degree (Gabriel, 1999a). There are many reasons that lead to the emergence of the disease, including the effects of oxidative stress or the presence of genetic factors where studies indicate that the injury is associated with the presence of antigens type HLA–DR or dysfunction of the immune system Rheumatoid arthritis is an autoimmune disease. The disease is characterized by inflammation of the synovial membrane and the joints of synovial and early indications of the disease is swelling and joint pain between the stages followed by the injury of large joints, especially the joints of the knee and elbow and ankle children under the age of 17 years may be infected with the Juvenile Rheumatoid arthritis disease And that females under the age of 8 years are more vulnerable to infection and that some of them may have antibodies to the nucleus and the factor of rheumatic serum (Robbins, 2001; Gabriel, 1999a; Rubins, 2005).

Studies indicate an increase in the mortality rate in patients with rheumatoid arthritis due to gastroenteritis, cardiovascular disease, respiratory diseases and blood diseases and increased prevalence and high mortality are evident in patients with limited annual income and low cultural level (Robbins, 2001; Rubins, 2005; Arvidsan, 2003; Callahan and Pincus, 1999). Rheumatoid arthritis is associated with the dysfunction of some cytokines especially the tumor necrosis factor, which is a multifunctional cytokine with inflammatory effects and mediate several autoimmune diseases. Some cytokines such as interleukin-1 and tumor necrosis factor play a role in the inflammation that causes the destruction of joint and bone during the disease of rheumatoid arthritis where the factor of necrosis of the alpha tumor is the factor, which contributes to rheumatoid arthritis where its biological efficacy comes from its association with special receptors called the TNF receptors (TNFR) Type I and II. There are Single Nucleotide Polymorphism, which gets in the gene factor alpha necrosis factor. Some are located within the coding region lead to making the gene silent, which does not encode and others fall within the area of the promoter, which leads to a defect in the process of gene expression, polymorphism or genetic variation for the promoter region. Gene is an alpha necrosis factor where it is associated with susceptibility to disease break the joints and produce autoantibodies for rheumatoid arthritis in many populations, alpha-necrosis factor has a key role in inflammation by stimulating the production of (expression) of other inflammatory molecules such as chemotactic cytokines and adhesion factors (Karray *et al*, 2011).

The aim of this study

The aim of this study was to use molecular techniques to verify the association between the polymorphism of the alpha-alveolar factor at site (G-308A) and its relationship with rheumatoid arthritis disease in a group of Iraqi patients infected with this disease using the technique of Tetra-Primer ARMS-PCR. There are many researchers in the world have studied the relationship between the factor of necrosis of the tumor and rheumatoid arthritis using various techniques such as technique PCR-RFLP and ARMS-PCR, which takes time, effort and high cost but did not find a study dealing with the use of Tetra-Primer ARMS-PCR technique In detecting a mutation (G-308A).

MATERIALS AND METHODS

Used specimens

Patients Group : This group included 100 patients suffering from rheumatoid arthritis from different parts of Saladin Governorate (48 males) and (52 females) between the ages of 30-70 years, based on the clinical symptoms and according to the diagnosis of specialist doctors, were confirmed to be infected with the disease after Conduct laboratory tests such as ESR, CRP and RA.

Control group : This group included healthy people as a comparison group. There were 50 randomly assigned patients aged between 30-45 years after being confirmed not to have rheumatoid arthritis.

Biochemical test

1. **ESR :** This test is performed in a manner Westrengren, where it is placed 1.6 ml from Blood with 0.4 ml of sodium citrate as an anticoagulant, then the sample is well dosed and then pulled by a Westrengren tubeto zero and fix the tube in the special tube holder in a vertical position and away from any vibration and then read the rise of the plasma column of the spectacular above the level of cells left after an hour.
2. **Rheumatoid factor (RF) :** It is tested using a Kit made from the company and is placed with a drop of serum with a drop of Globulin Reagent on a test slide Mix the mixture well to observe coagulation within two minutes.
3. **CRP Test :** C- reactive protein through segments specific to this test prepare with the Kit.

Genomic DNA isolation

The genomic DNA was isolated from the whole blood

Table 1 : Represents the primers used in the ARMS-PCR technique

	Name of primers	Sequence	Total concentration of primers	Annealing Tm	Size of bands (bp)
1	Forward inner primer (G allele)	5'TGGAGGCAATAGGT TTTGAGGGGCAGGA	10 Pml/il	68	224
2	Reverse inner primer (A allele)	5'TAGGACCCTGGAG GCTGAACCCCGTACC	10 Pml/il	68	154
3	Forward outer primer (5'–3')	5'ACCCAAACACACG CCTCAGGACTCAACA	10 Pml/il	68	323
4	Reverse outer primer (5'–3')	5'AGTTGGGGACACGC AAGCATGAAGGATA	10 Pml/il	68	323

and the way it was mentioned (Ali, 2008).

Determination of the amount of DNA

DNA concentration was estimated using a device Nanodrop. The concentration was calculated on the assumption that one optical density of 260 nm corresponds to 50 µg/ml of DNA. The different samples were diluted using sterile distilled water to reach a concentration of 50 ng/µl.

Preparation of samples for technical ARMS-PCR

To determine the polymorphism of the tumor necrosis factor at site (G-308A) Applied ARMS-PCR technical, using four specialized primers designed for this purpose which give packages of different sizes equipped with powder (Lyophilized) formulations by a company Bio basic.

The interactions were conducted ARMS-PCR technique Referring to Melanie *et al* (2006) by using AccuPower PCR premix Kit Processed by the company Bioneer in Korea. According to the attached instructions, mix all components of the reaction ARMS-PCR using a micro pipette then centrifuge for 10 seconds in a device Microcentrifuge to precipitate the reaction solution droplets located on the tube wall and place the tubes in the thermocycler device carefully to achieve the reaction and at a temperature of 68^0C. After the reaction time, the tubes were lifted from the thermocouple 5ìl were then withdrawn from each tube and placed in the electrolyte gel and then electrophoresis. The dye of the gel with the dye of the ethidium bromide lasted from 45-60 minutes with stirring, finally using a device Gel Documentation System for Photography.

The general equation for this test was :

X2 = £ (Observed values - Expected values)2 / Expected values.

RESULTS AND DISCUSSION

Clinical characteristics and biochemical tests

Rheumatoid Arthritis has spread throughout the world with a variety of severity and clinical signs among population groups in Iraq. This disease has become a common disease and its incidence has increased over time.

This study is consistent with other studies conducted in Iraq and differs with what is mentioned in the countries of the world where the prevalence of the disease in the fourth decade of life and this is due to differences in genetic and environmental factors (Al-Obeidy and Abdullah, 2011). The study also indicated that all patients with rheumatoid arthritis had a high rate of red blood cell deposition ESR, in levels of interactive protein CRP. In comparison with the healthy group, in addition to giving the rheumatoid factor RF a positive value of 90% compared to the healthy group.

Isolation of Genomic DNA

Adequate amounts of DNA were obtained from the blood using the DNA isolation method described previously (Ali, 2008). To obtain DNA in less time plus get a proper concentration and purity range from 1.4-1.8.

Results of Tetra-Primer ARMS-PCR Technical

This technique was used to detect the genetic patterns of the tumor necrosis gene at site (G-308A) and its relationship with rheumatoid arthritis among a group of Iraqi patients infected with the disease and in fact there are many researchers who studied the relationship between the gene and rheumatoid arthritis, but used other techniques such as technique PCR-RFLP and ARMS-PCR. But we did not find any study used technique Tetra-Primer ARMS-PCR in gene detection (G-308A) .

After completion of electroplating Tetra-Primer ARMS-PCR technique for 100 samples of GenomicDNA

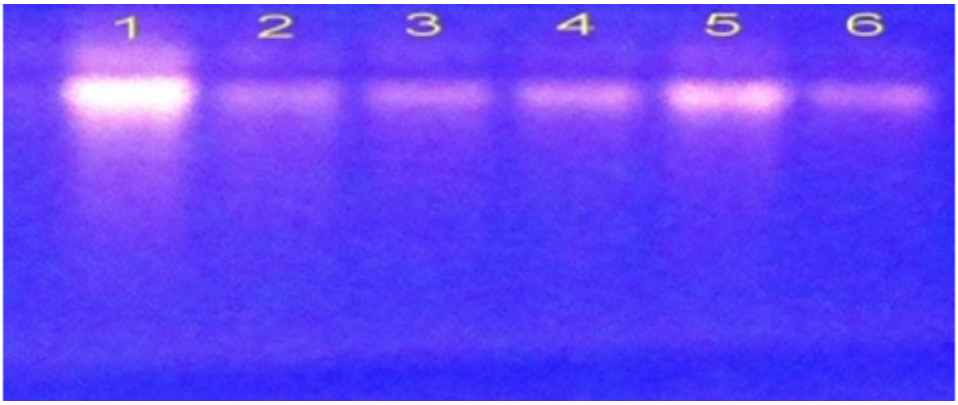

Fig. 1 : Some of the Genomic DNA samples extracted from blood and migration on the Agarose gel at a concentration of 1%.

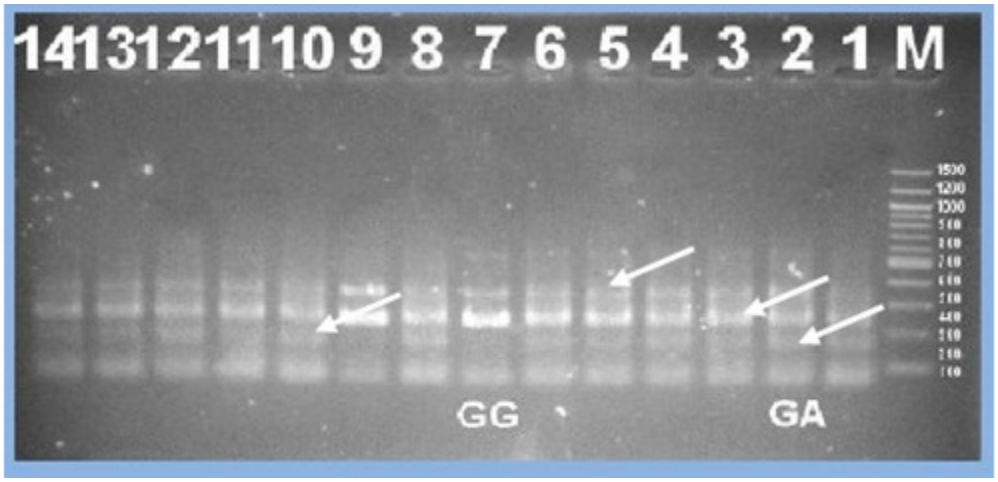

Fig. 2 : The result of electrophoresis Tetra-Primer ARMS-PCR technique.

Table 2 : Distribution of rheumatoid arthritis patients by age and sex compared to control group.

Age groups	Study group			
	Rheumatoid arthritis patients		Healthy people	
	The number	Percent	The number	Percent
20 <	8	8%	8	16%
20-29	9	9%	9	18%
30-39	24	24%	19	38%
40-49	17	17%	8	16%
50-59	20	20%	4	8%
60-69	16	16%	2	4%
70-79	6	6%	-	-
Females	84	84%	34	68%
Male	16	16%	16	32%
Total	100	100%	50	100%

for patients with rheumatoid arthritis and 50 Genomic DNA samples of Healthily people on the agarose gelat concentration 3%. The resulting packets of multiplication of all samples were shown where the larger master package and its molecular size shows 323 base pairs specific to the A allele in the case of three bands. This means that the sample is heterozygous, either in the case of the package of the A allele with the main band it will be Homozygote Of the natural type In the case of special band G allele with the main band being Homozygote of the mutant type as in Fig. 2.

The results of this study showed a high frequency ofG allele among the group of Iraqi rheumatoid arthritis patients this is consistent with Al-Rayes *et al* (2014) in Saudi community (Ozen *et al*, 2002) in Turkish community and Rodriguez-Carreon (2005) in Mexican community. The interpretation of this agreement and the difference is not clear but can be traced back to the ethnic difference and to the different environmental conditions between one community and the other to the interaction between genes and the environment where (Lee *et al*, 2007) suggested that race has a significant impact on the distribution of genotypes and frequently of alleles in patients with rheumatoid arthritis. Rheumatoid arthritis is also a complex disease that is affected by environmental and genetic factors in terms of its origin and severity. Therefore, the interaction between genetic and environmental factors plays a major role in determining the genotype and thus affecting the patient

(Al-Rayes *et al*, 2014).

The results of this study indicate that there is an association between TNF-308 polymorphism and clinical manifestations as a positive RF, High CRP and rate ESR this differs with what Al-Rayes *et al* (2014) found in Saudi community where Hyde (2000), Nemec *et al* (2008) reported a close association between the severity of rheumatoid arthritis and multiple forms of tumor necrosis gene at the site (–308) in American and Mexican community. The gene of the tumor necrosis factor can affect rheumatoid arthritis so that the relationship between genetic patterns and clinical characteristics can reflect different patterns of severity of the disease that affect the type of treatment used. The genetic basis of rheumatoid arthritis is the presence of genes within the histopathological complication complex HLA, which have a clear role in preparing for the disease and its severity, on the other hand there is a close link between the disease and women, where the proportion of women are more than three times the men (Al-Rayes *et al,* 2014).

Khanna *et al* (2006) found a correlation between polymorphism TNF-308 the development of damage in patients with American arthritis and pointed out that the correlation can depend on the genetic variables in the imbalance of the link between TNF-308A allele and DRB1* 0301. While, Nemec *et al* (2008) did not find any difference in the distribution of genetic patterns and frequency of TNF-308 alleles among Czech arthritis patients according to the division of the group RA according to the evolution of the disease has observed a significant difference in the distribution of genotypes, suggest that the genotype GG from TNF-308 polymorphism associated with the severity of arthritis in the Czech population has been mentioned by Schmeling *et al* (2006) a genotype of GA from TNF-308. It is associated with ulcers that are common in patients with German arthritis.

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