Serum Tumor Necrosis Factor Alpha and Gene Polymorphisms in Rheumatoid Arthritis Patients in Babylon Province, Iraq


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Abstract

Background: Rheumatoid arthritis (RA) is a systemic autoimmune disease affects 0.5-1% of the worldwide population, of unknown etiology, characterized by chronic inflammation of the synovial joints, hyperplasia, and overgrowth of synoviocytes, with the destruction of articular cartilage that can cause serious weakness and inability to work. Objective: The present study aims to investigate the possible association between tumor necrosis factor alpha (TNF-α) levels and (-308 G/A) TNF-α promoter polymorphism in patients with RA in Babylon Province. Patients and Methods: This study was designed as a case control. Forty-five (10 males, 35 females) patients with RA and forty-five (9 males, 36 females) apparently healthy persons as control with the compatible age and sex were enrolled in this study. Serum level of TNF-α and anti-cyclic citrullinated peptide antibodies (ACCPA) were measured using enzyme-linked immunosorbent assay (ELISA) method. Disease activity of RA patients was assessed using the Disease Activity Score-28 (DAS28). The frequency of TNF-α (-308 G/A) gene polymorphism was determined using polymerase chain reaction, restriction fragment length polymorphism (PCR-RFLP) technique. Results: Present study finds significant high levels of serum TNF-α and ACCPA in patients with RA in comparison with healthy controls. The genotype of (-308 G/A) TNF-α gene promoter polymorphisms the GG genotype was 60% in RA patients and 42.2% in control group, while the GA genotype was 40% in RA patients and 53.3% in control group, whereas, the AA genotype was 0% in RA patients and 4.4% in control group. Correlation between TNF-α levels with both of DAS-28 and ACCPA in RA patients found to be a significant positive correlation proposes a probable role of TNF-α in RA pathogenesis. Statistical analysis showed no significant difference in the genotype frequency of the TNF-α (-308 G/A) gene promoter polymorphisms between two groups. Conclusions: The results of the present study were shown that TNF-α (-308G/A) gene polymorphism had not associated with a risk factor of RA in Babylon patients and this SNP do not affect the serum level of TNF-α in RA patients.

Keywords: Rheumatoid arthritis, TNF-α, -308 G/A gene polymorphism, ACCP Antibody, PCR-RFLP, Iraq.

Introduction

Rheumatoid arthritis is persistent systemic inflammatory is the most common type of autoimmune sickness causes morning joint stiffness, pain, fever and damage to function. thus lead to the transformation in the joint as joint inflammatory that resemble rheumatic fever [1].

The RA is initially associated with inflammation of synovial joints. RA mostly effect all peripheral joints although the most commonly affected joints are included hands, feet, and knees [2]. RA may develop the risk of several other diseases such as intestinal pathologies, pulmonary dysfunction, cardiovascular disease, and kidney disorders with a significant rise in the risk of early death [3].

The epidemiology of RA is affecting 0.5-1 % of the worldwide and affects females more frequently than male in the ratio (3:1) [4, 5]. RA may appear at any age. RA has a prevalence of 20-50 cases each 100.000 persons / year [6].

Arthritis is the most common health problem in the United States population, affected over than 46 million people and
resulting in disability for 19 million populations [7].

The peak age of onset worldwide is between 65 to 75 years in men and 55-64 years in women [8]. There is no description of the reason for the geographic variation [9]. There are no predictions for the future incidence of rheumatoid arthritis, although some studies are reported a decrease in incidence and a rise in the average age of onset [10,11]. A proposed reason for the decrease in RA is hygiene [10]. There is multi-factorial that causes RA but they still consider unidentified etiology. Genetic, environmental factors and deregulated immune responses in the body found to play a part in the stimulation of the disease activity [12].

The disease is defined by increased expression of proinflammatory cytokines such as Interleukin (IL)-1, IL-2, IL-6, IL-17, Interferon-gamma (INF-γ), and Tumor necrosis factor-alpha (TNF-α). Most of these cytokines can be detected in synovial fluid from RA patients [13]. TNF-α is an inducible cytokine with a broad range of proinflammatory and immunostimulatory actions. This cytokine plays an important role during the pathogenesis of RA. It initiates the inflammatory response leading to edematous joint and subsequent bone destruction during the development of disease [14].

The location of TNF-α gene on chromosome number 6p21.3, TNF-α is a cytokine that is produced by many cell types, but macrophages and monocytes are the primarily associated with the synthesis of this TNF-α protein [15]. When the TNF-α polymorphisms in promoter region occur, this lead to increase the TNF-α production, which in turn may have an impact on inflammatory responses, disease expression and response to therapy. There is many of SNPs in TNF-α region are reported to be associated with RA susceptibility in various populations [16, 17].

The present study aims to estimate the levels of TNF-α and ACCPA in patients with RA and healthy controls in the case-control study, as well as to investigate the relationship among TNF-α and ACCPA with DAS-28 in RA patients. The present study also aims to investigate the possible association between TNF-α levels and TNF-α (-308 G/A) gene promoter polymorphism in patients with RA in Babylon Province, Iraq.

**Materials and Methods**

**Ethical Issues**

The ethical issues in the present study are based on the following:

- The acceptance of the Scientific Committee of Department of Biochemistry at the College of Medicine University of Babylon.
- The Ethical Committee in College of Medicine, University of Babylon.
- The acceptance of the Ethical Committee of Babylon General Directory of Health.

All persons participate in this study were understood the objectives of study and signed an informed consent.

**Patients**

The sample size of patients group in the present study was selected according to sample size equation. This case control study was included 45 patients (10 males and 35 females) with RA with mean age (47.75 ±7.67) years, diagnosed by a specialist physician when they attended to Rheumatoid Unit at Merjan Teaching Medical City in Hilla City, and according to the American College of Rheumatology (ACR) criteria in the year 1987 and according to 2010 / European League Against Rheumatism (EULAR) classification criteria for RA [18].

The activity of RA disease was assessed by using DAS-28 measurement. The complete history was reported and a comprehensive questionnaire has been filled. Control group consist of 45 (9 males and 36 females) apparently healthy persons with mean of age (47.46 ± 7.76) years. They were selected from medical staff of the same medical city. The period of this study was extended from first of August / 2016 to the first of July / 2017.

**Exclusion Criteria**

The persons with any type of acute or chronic disease as well as persons who were obese, smokers and pregnant women were excluded from the present study.

**Inclusion Criteria**

Individuals were established with RA within age from 35-60 years were included in the
present study as patients group, in addition to apparently healthy persons as control group within age from 35-59 years.

Methods

Measurement of Disease Activity Score (DAS-28)
The DAS-28 of RA patients were estimated by Van Riel formula [19].

Measurement of Serum Tumor Necrosis Factor Alpha (TNF-α)
Quantitative determination of TNF-α in the current study was done by using Bio base® ELISA kit.

Measurement of Serum ACCP Antibodies
Quantitative determination of ACCPA in the current study was done by using Bio base® ELISA kit.

Measurement of Blood Rheumatoid Factor (RF)
The RF in the study groups was measure by using Spin react® RF-latex agglutination test kit.

Measurement of Blood C reactive Protein (CRP)
The CRP in the current study was determined by using Agappy® latex-promoted nephelometry kit.

Determination of (-308) Promoter Codon Polymorphism of TNF-α Gene
The PCR/ RFLP was used to investigate the (-308 G/A) TNF-α gene polymorphism promoter codon in RA patients and control groups.

DNA Extraction and TNF-α Genotyping
The Favorgen- Taiwan Mini Kit for Genomic DNA gives a good method for the purification of DNA from blood samples. Enzymatic amplification was done by PCR using Master Taq polymerase enzyme and hybrid thermal cycler. The amplification process was done as proposed by E. F. Karray et al. [20] by utilizing two primers (Promega®).

The forward and reverse primers are
5’AGGCAATAGGTTTGTAGGCCCATT-3’
5’TCCCTCCCTGCTCGATTCCG-3’

The reaction mixture of PCR (25 µl) consist of 12.5 µl, PCR Master Mix [10 × PCR buffer, 4 mM MgCl2, 0.5 Taq DNA polymerase 0.4 mM dNTPs (dTTP, dCTP, dATP and dGTP)], 1 µl of both primer, 3 µl of extracted DNA and 7.5 µl sterilized nuclease-free water.

The reaction was performed with the following cycles
One cycle 5 min at 94 °C, 30 seconds at 94°C for denaturation, 30 cycles 35 seconds at 60°C for primer annealing, 1 minute at 72 °C for template elongation, 10 minutes at 72°C for final elongation. After that, the amplified products were digested with 5 units Fast Digest NCOI restriction enzyme at 37°C for about (2-4 hours). The digested products were then detected on (6%) of polyacrylamide gel electrophoresis technique (PAGE) and visualized on (UV transilluminator) [21].

Results

Results of the present study and other study were shown no difference between males and females in the most parameters involved [13]. Therefore, categorization of participants did not depend on the gender, as shown in Figure 1.
Also, this study showed no significant differences in the mean age when compared between patients with RA and healthy control group, as shown in Table (1).

### Table 1: The Mean of Age of Study Group

<table>
<thead>
<tr>
<th>Study group</th>
<th>No.</th>
<th>Male</th>
<th>Female</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>45</td>
<td>10</td>
<td>35</td>
<td>47.75 ± 7.67</td>
<td>0.86</td>
</tr>
<tr>
<td>Control group</td>
<td>45</td>
<td>9</td>
<td>36</td>
<td>47.46 ± 7.76</td>
<td></td>
</tr>
</tbody>
</table>

The DAS-28 in the present study was shown no significant differences between means of DAS-28 according to gender among patients with RA, as shown in Table (2).

### Table 2: DAS-28 of Patients with Rheumatoid Arthritis

<table>
<thead>
<tr>
<th>Gender</th>
<th>No.</th>
<th>Mean ± SD</th>
<th>T-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>10</td>
<td>4.02 ±0.89</td>
<td>0.037</td>
<td>0.971</td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>4.00 ± 0.86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The serum TNF-α concentration of RA patients groups significantly elevated than control groups, as shown in Table (3).

### Table 3: Tumor Necrosis Factor Alpha of Patients and Control Group

<table>
<thead>
<tr>
<th>Study group</th>
<th>No.</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>45</td>
<td>81.31 ±28.14</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Control group</td>
<td>45</td>
<td>24.52 ± 6.64</td>
<td></td>
</tr>
</tbody>
</table>

In the present study, serum ACCPA concentrations of patients with RA were found to be significantly greater than control groups, as shown in Table (4).

### Table 4: Anti-Cyclic Citrullinated Peptide Antibody in Patients with RA and Healthy Control

<table>
<thead>
<tr>
<th>Study group</th>
<th>No.</th>
<th>Mean ± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>45</td>
<td>27.31 ±11.2</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Control group</td>
<td>45</td>
<td>5.66 ± 2.23</td>
<td></td>
</tr>
</tbody>
</table>

The Correlations among TNF-α levels and DAS-28, TNF-α levels and ACCPA, and DAS-28 and ACCPA in RA patients were investigated and found to be significant positive correlations. In the present study, CRP of patients with RA found to be positive (50% of males and 100% of females). The present study was found RF of patients with RA positive in 69.3% and negative in 30.7 % in all cases with RA patients. After that, the amplification products by PCR were separated by 3% agarose gel electrophoresis technique and stained with ethidium bromide dye. The PCR product length was (107 bp), as shown in Figure (2).

Figure 2: The Amplification and PCR Product (107bp) on 3% Agarose Gel Electrophoresis. Lane M, DNA ladder 50bp (128ng/µl) and other lanes represent PCR products (107bp)
The yields of amplification after that digested by using a specific restriction enzyme NcoI of -308 G/A TNF-α gene promoter codon via PCR-RFLP method were shown two alleles (G and A) with three genotypes:

They appear one band with 107 bp and AA genotype mean the digestion not occur, GG was digested with restriction enzyme NcoI into two bands 87 and 20 bp, and AG has three bands 107, 87, and 20 bp, as shown in Figure (3).

Figure (3) Amplification and Digestion by Restriction Enzyme of -308 G/A TNF-α Gene Promoter Codon using 6% Polyacrylamide Gels Electrophoresis. The Lane (M) DNA Ladder 50 bp(128ng/µl) and Lanes (1-11) for Patients and (12-14) for Control

Results of current study found no significant association of TNF-α (-308 G/A) gene promoter polymorphisms of susceptibility to RA, the current study also found genotype of GG 60% in RA and 42.2% in control group, the GA genotype frequency 40% in RA and 53.3% in control group, the AA genotype frequency 0% in RA group and 4.4% in control group, while the AA fewer genotype frequency in all subjects of present study, as shown in Table (5).

<table>
<thead>
<tr>
<th>Model</th>
<th>Genotype</th>
<th>Control</th>
<th>Patients</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codominant</td>
<td>G/G</td>
<td>19 (42.2%)</td>
<td>27 (60%)</td>
<td>1.00</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>24 (53.3%)</td>
<td>18 (40%)</td>
<td>0.53 (0.23-1.23)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>2 (4.4%)</td>
<td>0 (0%)</td>
<td>0.142</td>
<td>0.006-3.12</td>
</tr>
<tr>
<td>Dominant</td>
<td>G/G</td>
<td>19 (42.2%)</td>
<td>27 (60%)</td>
<td>1.00</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>G/A-A/A</td>
<td>26 (57.8%)</td>
<td>18 (40%)</td>
<td>0.49 (0.21-1.13)</td>
<td></td>
</tr>
<tr>
<td>Recessive</td>
<td>G/G-G/A</td>
<td>43 (95.6%)</td>
<td>45 (100%)</td>
<td>1.00</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>2 (4.4%)</td>
<td>0 (0%)</td>
<td>0.191</td>
<td>0.009-4.097</td>
</tr>
<tr>
<td>Over dominant</td>
<td>G/G-A/A</td>
<td>21 (46.7%)</td>
<td>27 (60%)</td>
<td>1.00</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>24 (53.3%)</td>
<td>18 (40%)</td>
<td>0.58 (0.25-1.35)</td>
<td></td>
</tr>
</tbody>
</table>

Results of current study concerning allele association of TNF-α (-308 G/A) polymorphism of patients with RA were shown that no significant difference in genotype and allelic frequencies in Babylon people after comparing between RA patients and healthy control group, as shown in Table (6).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control</th>
<th>Proportion</th>
<th>Patients</th>
<th>Proportion</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>62</td>
<td>0.69</td>
<td>72</td>
<td>0.8</td>
<td>1.8 (0.913-3.575)</td>
<td>0.08</td>
</tr>
<tr>
<td>A</td>
<td>28</td>
<td>0.31</td>
<td>18</td>
<td>0.2</td>
<td>0.55 (0.280-1.096)</td>
<td></td>
</tr>
</tbody>
</table>
The result of the present study was found a negative non-significant association between TNF-α levels and genotypes in RA patients the P-value \( = (0.6) \). Also, the result of the present study was shown a negative non-significant association between DAS-28 activity and TNF-α (-308 G/A) gene promoter polymorphism in patients with RA the P-value \( = (0.75) \). The results of the current study were also found a negative non-significant correlation between ACCP antibody and TNF-α (-308 G/A) gene promoter polymorphism of patients with RA the P-value \( = 0.56 \).

**Discussion**

The present study was found the serum TNF-α concentration of RA patients groups significantly, elevated than control groups, this result is agreed with Hadinedoushan et al. study that recorded the levels of serum TNF-α concentration in cases of RA were significantly greater than the healthy control group [21, 22]. TNF-α has the vital key role in pathogenesis events of RA. This cytokine is formed in elevated concentration through many of cells, for example, monocytes and T cells in case of RA infection [23]. An elevated level of TNF-α is present in the synovial fluid and serum of RA patients [24]. Therefore, it has been established that countervail monoclonal antibody versus TNF-α lead to decrease the production of another’s pro-inflammatory cytokine, for example, IL-1 and GM-CSF in the synovial fluid of RA [25].

The result of the current study disagreed with Beyazal et al. study which recorded the mean serum IL-17 and TNF-α concentration was not significantly different among RA patients and healthy group [26]. The correlation between TNF-α and ACCP levels in the present study were investigated and shown a positive and significant correlation in patients with RA groups \( (P<= 0.001, r = 0.542) \) the result of the current study is agreed with Clavel et al.[27] study that reported ACCPA contain immune complexes stimulate TNF-α production through human macrophages via binding of Fc γ RII α at the surface of macrophage, this means that when ACCP levels are elevated this will lead to induce TNF-α production [28].

The current study was found a positive and significant correlation between serum TNF-α levels and DAS-28 in patients with RA disease. The DAS-28 is usually used in the measure of disease activity in RA and help patients in how controlled his RA and whether therapy is effective or not [29]. This result of the current study is agreed with Nemec et al. [30] study which found the elevated level of TNF-α in synovial fluid of RA patients indicated a positive correlation between the serum TNF-α level and disease activity.

Results of current study found no significant association of TNF-α (-308 G/A) gene promoter polymorphisms of susceptibility to RA also, the current study found genotype of GG 60% in RA and 42.2% in control group, the GA genotype frequency 40% in RA and 53.3% in control group, the AA genotype frequency 0% in RA group and 4.4% in control group, the GG more genotype frequency, while the AA fewer genotype frequency in all subjects of present study is agreed with Hadinedoushan et al.[22] study, that found GG more genotype frequency and the AA fewer genotype frequency at Iran population. The current study is also agreed with Deepali G. et al.[31] study that reported no significant association of TNF-α -308 bp G/A polymorphism with susceptibility to RA in North Indian people. There are no associations have also been found in South East Asians [32].

As well as agreed with Song et al. [33] study that reported the TNF-α (-308 A/G) polymorphism positive risk factor for RA in Latin Americans, but not in the European, Arab, or Asian populations. The present study disagreed with Camelia A et al. [34] study that carried out on Egypt population that reported the TNF-α -308 G/A gene polymorphism positive association in development of RA. Results of current study concerning allele association of TNF-α (-308 G/A) polymorphism of patients with RA were shown that no significant difference in genotype and allelic frequencies in Babylon people after comparing between RA patients and healthy control group.

The allele frequency in the current study of A allele found no significant difference and low frequency of this allele in patients, where the A allele count was 18 and proportion 20% while the A allele in control was 28 and proportion 31% \( (P=0.08) \). Results were shown that the allele frequency of G allele was the major allele frequency in patients where it was found that the G allele counts 72 and
proportion 80%, while the G allele in control group was count 62 and proportion 69% (P=0.08). The current study is agreed with Nemec et al.[35] study that found no significant difference in genotype and allelic frequencies of TNF-α (−308) polymorphism in Czech population with and without RA. As well, the current study disagreed with Hussein et al.[36] study that found a significant association of TNF-α -308 alleles with RA. The exact cause of the difference in the distribution of alleles and genotypes of TNF-α (−308) polymorphism in RA patients in different ethnic groups is far from clear [37].

Conclusion

The results of the present study were shown that TNF-α (-308G/A) gene polymorphism had not associated with a risk factor of RA in Babylon patients and this SNP do not affect the serum level of TNF-α in RA patients. Further prospective studies with large sample sizes with different ethnicities are required to confirm our results. In addition, multiple SNPs should be measured in future studies.

References


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