Association of TNF-α Promoter Polymorphisms and Some Inflammatory Cytokines with Susceptibility to Rheumatoid Arthritis in Saudi Population

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Abstract
Background: Tumor Necrosis Factor-α (TNF-α) is coded and regulated by TNF-α gene which is implicated in the predisposition of Rheumatoid Arthritis (RA). This study aims to detect TNF-α (G-308A) promoter polymorphism and evaluate its association with susceptibility to Rheumatoid Arthritis.

Methods: In the present study, 50 patients with RA and 50 healthy individual were included and evaluated for the C-reactive protein, rheumatoid factor, and TNF-α were evaluated by ELISA, Erythrocyte Sedimentation Rate (ESR) by Westergren method and for TNF-α -308 G > A polymorphism by polymerase chain reaction with amplification refractory mutation system (PCR-ARMS).

Results: The CRP, RF, ESR and TNF-α were significantly elevated in RA patients relative to controls. The serum level TNF-α was also significantly elevated in female patients and in patients ≥ 50 years. Analysis of TNF-308 gene polymorphism revealed that GG genotypes were more common in RA than in the controls and that GG genotype may be a risk factor to RA. The G allele was more frequent in RA than in the control. Elevated TNF-α serum levels were significantly associated the GG genotype and functional disability in RA patients.

Conclusion: TNF-α promoter 308 polymorphism GG genotype may be considered as a risk factor for RA and the TNF-α serum level was significantly related to the functional disability in the disease.

Keywords
Rheumatoid arthritis, Gene polymorphism, Tumor Necrosis Factor (TNF-α)

Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory condition that results in joint deformity and function loss within10 years after initiation [1]. It is characterized by inflammation of the synovial membrane and damage of the joint by the immune cell results in severe arthritis with a progressive deterioration of the joint cartilage [2]. It has a major negative effect on the ability to perform daily activities, including work which household tasks, and health related quality of life [3]. Epidemiological evidence exists that genetic factors are related to an increased risk of RA [4]. RA is known to be a multifactorial disorder, triggered by the combination of hereditary and environmental elements that lead to its development and appearance. Genetic susceptibility, sex and age, smoking, infectious agents, biochemical, nutritional, socioeconomic, and ethnic factors are risk factors for the disease. Most of these variables are likely associated with both the onset and severity of the disease [5].
RA is the commonest autoimmune disease. It is related to the alteration in cytokine production that may have a pathogenic function in the development of RA [6]. Cytokines are molecules that secreted by immune cells and involved in cell-to-cell communication during cell injury, infection, and inflammation, and it is a vital component of immune system communication [7]. The inflammatory cytokines found in RA patients were higher in the joints than in the blood, with most of them being pro-inflammatory. Such cytokines include IL-1, TNF-α, IL-6, IL-15, IL-16, IL-17, IL-18, interferon (IFN)-γ, a stimulating factor for granulocyte macrophage-colony, contributing to local joint destruction and the systemic effects of the disease [8]. Tumor necrosis factor (TNF-α) is a strong pro-inflammatory cytokine that stimulates cytokine production, increases the expression of adhesion molecules, increases neutrophil activation, and acts as a co-stimulant for T-cell activation and antibody development; TNF-α abnormalities have been reported in RA [9]. The release TNF-α and IL-1 have many harmful effects on the cartilage and bone [10]. TNF-α is a pleiotropic cytokine that plays an important role in mediating various immune functions including inflammation [11], regulation of apoptosis and necrosis [12], and induction of cytotoxicity [13]. TNF-α is able to initiate a number of immune reactions by binding to type I and type II receptors found on the membrane with a molecular weights 55 and 75 kDa [14]. Type I TNFα receptors (TNFRI) are more common and expressed on all types of cells, as compared to Type II TNFα receptors (TNFRII) expressed mainly on the immune system cells [15].

TNF-α is coded and regulated by TNF-α gene, which is located in chromosome 6 [9], within the class III region of major histocompatibility [16]. Several studies analyzed the implication of TNF-α gene with predisposition to Rheumatoid Arthritis [9]. Inter individual variations in the TNF production in healthy controls have been observed with high and low producer phenotypes indicating a significant genetic involvement to regulation of the TNF-α synthesis [17], these findings propose that polymorphism in the TNF-a regulatory region might influence its production. A number of single nucleotide polymorphisms (SNPs) within the promoter of the TNF-a gene has been identified, among these common polymorphisms in the promoter, a G-to-A transition at position 308 (rs1800629) has been most widely analyzed [17].

TNF-α overproduction contributes to various diseases such as inflammatory intestinal disease and RA [2]. In the treatment of chronic rheumatoid disease, TNF-α inhibitors play a significant role [18]. In TNF-α, a genetic variation on -308 leads to two allelic variants in which guanine (G) defines the common variant and adenine (A) defines the less common one. TNFα-308 A-allele reveals increased gene transcription relative to G. It has been shown to produce 6-7 fold higher levels of TNF-α transcription [19]. This study was carried out to detect TNF-α (G-308A) promoter polymorphism and evaluate its association with susceptibility to Rheumatoid Arthritis.

Methods

This study is a case-control carried out at the female section of College of Applied Medical Sciences, Taif University. The study included 50 patients with RA and 50 healthy individuals of both genders, selected according to the American College of Rheumatology (AC) [22], and the Lequesne index [23]. Systemic lupus erythematosus, Mixed connective tissue disease, Dermatomyositis, and Scleroderma. Functional disability in RA was assessed in all patients according to Kellgren score [21], the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) [22], and the Lequesne index [23].

Preparation of blood samples

A 10 ml peripheral venous blood sample was collected from all included subjects. Five milliliters of each obtained sample was withdrawn in EDTA tubes for DNA extraction and ESR determination. The remaining sample was left for 1 hour in a serum separator collection tube to clot at room temperature and centrifuged at 3000 rpm for 5 minutes. The separated serum was obtained and stored in -20 °C until analysis. CRP and RF levels were estimated in serum samples from RA patients and control subjects using Human C Reactive Protein ELISA Kit (CRP), abcam, Cambridge, MA, USA (Cat No. ab99995), and rheumatoid factor (RF), ELISA Kit, MyBioSource, Inc. San Diego, CA 92195-3308, USA (Cat No. MBS262327), respectively. ESR was determined using Westergren method. TNF-α level was estimated in serum samples from RA patients and control subjects using Abcam Human TNF alpha ELISA Kit, Cambridge, MA, USA, (ab181421) according to the manufacturer’s protocol.

Genomic DNA extraction

Genomic DNA was extracted and purified using the QIAamp DNA mini kit (Qiagen CA, USA) from the peripheral blood collected in tubes containing EDTA from rheumatoid arthritis patients and controls. Purified DNA was kept at -80 °C until used for genotyping.

TNF-α -308 G > A promoter polymorphism detection by (PCR-ARMS)

Genotyping of TNF-α -308 G > A was performed us-
ing the polymerase chain reaction with amplification refractory mutation system (PCR-ARMS) as described by Perry, et al. [24]. The PCR amplification was carried out using recombinant Taq polymerase master mix (Dream taq green, code number k1081, LOT: 00643300) (Thermo Fisher Scientific Ballics UAB, V A Cracino 8, LT-002241 Vilnius, Lithuania) in a 25 μl total volume TNF-α Primer sequences were as follows: TNF-α G forward primer: 5′-ATA GGT TTT GAG GGG CAT GG-3′; TNF-α A forward primer: 5′-AATA GGT TTT GAG GGG CAT GA-3′; and common reverse primer, 5′-TCT CGG TTT CTT CTC CAT CG-3′. Reaction conditions were carried out at 95 °C for 1 min followed by 10 cycles of 95 °C for 15 s, 65 °C for 50 s using heated lid thermal cycler (Mastercycler pro, Eppindorf, Germany), and 72 °C for 40 s and then 20 cycles of 95 °C for 20 s, 56 °C for 50 s, and 72 °C for 50 s. The amplified PCR products were analyzed by 2% agarose gel and ethidium bromide staining followed by ultraviolet visualization. The PCR product for TNF-α-308 was detected at 184 bp. A positive control was included in the PCR assay by amplification of the human growth hormone (HGH) gene.

Statistical analysis

Data were collected, tabulated and analyzed using the computer program SPSS version 22.0. In this study, qualitative data were presented by numbers and percentage and quantitative data were presented as mean and SD. For comparing qualitative variables, chi-square (χ²) test was used. A p-value of < 0.05 was considered significant and Odd Ratio (OR) was used and 95% Confidence Interval (C.I.).

Figure 1: Levels of CRP (A), RF (B), ESR (C), and TNF-α (D) in RA patients and the controls. (A) The difference of CRP levels in patients and controls was statistically significant (t = 15.58, P < 0.0001); (B) The difference between in RF serum levels among RA patients and controls was statistically significant (t = 19.58, P < 0.0001); (C) The difference between RA patients and the controls was statistically significant (t = 16.06, P < 0.0001); (D) The serum level TNF-α in RA patients was statistically significant (t = 20.92, P < 0.0001), compared to control group.
Results

The present study included 50 patients with rheumatoid arthritis (RA) and 50 healthy individuals. The age of the patients ranged 41 and 66 years while in the controls it ranged between 41 and 68 years. It included 11 male patients and 39 females, 14 male and 36 females of healthy individuals Table 1.

Creatine protein

The C-reactive protein level (CRP) in RA patients ranged between 10.3 and 55.6 mg/L with a mean value ± SD of 30.02 ± 11.48 while in the controls it ranged between 1.01 and 8.12 with a mean ± SD of 4.32 ± 2.1. The difference of CRP levels in patients and controls was statistically significant (t = 15.58, P < 0.0001), Figure 1A.

Rheumatoid factor (RF)

Analysis of serum level of RF in patients and controls. In RA patients the serum level of RF ranged between 15.21 and 55.37 IU/ml with a mean and SD value of 31.04 ± 10.64 IU/ml. meanwhile in the controls it ranged between 0.19 and 4.02 IU/ml with a mean and SD value of 1.475 ± 0.93, the difference between in RF

| Table 1: Age and Sex of RA patients and controls. |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Patients                                      | Controls        |
| No %                                          | No %            |
| Age < 50                                      | 14 28           | 16 32           |
| ≥ 50                                          | 36 72           | 34 68           |
| Sex Male                                      | 11 22           | 14 28           |
| Female                                        | 39 78           | 36 72           |

Erythrocyte sedimentation rate (ESR)

In patients with RA the ESR ranged between 35 and 128 mm/hr with a mean ± SD value of 72 ± 28 mm/hr. In control individuals ESR ranged between 1 and 13 mm/h with 7.68 ± 2.97 mm/h as a mean ± SD value. The difference between RA patients and the controls was statistically significant (t = 16.06, P < 0.0001), Figure 1C.

Tumor necrosis factor (TNF-α) levels in RA patients and controls

In RA patients the serum level TNF-α was ranged between 22.5 and 60.15 pg/ml with a mean ± SD value of 38.19 ± 12.34 pg/ml. In the control group, it ranged between 0.001 and 4.65 pg/ml with a mean ± SD value of 1.42 ± 1.47 pg/ml. The difference between both groups was statistically significant (t = 20.92, P < 0.0001), Figure 1D.

TNF-α serum level was found to be correlated with the patients’ age in RA. In patients less than 50 years the serum level ranged between 22.5 and 28.4 pg/ml with a mean and SD value of 24.9 ± 1.7 pg/ml. In patients with age more than 50 years it ranged between 28.9 and 60.2 with a mean ± SD value of 44.5 ± 9.9 pg/ml. The difference between both age groups was statistically significant (t = 7.837, P < 0.0001), Figure 2A.

The serum levels of TNF-α were assessed in RA patients according to their sex. In males the serum level ranged between 25.3 and 30.9 pg/ml with a mean and SD value of 28.1 ± 1.9 pg/ml. In females it ranged between 22.5 and 60.2 pg/ml with a mean ± SD value of 41.03 ± 12.6

Figure 2: Serum level of TNF-α in patients with RA according to their age (A) and their sex (B). (A) The serum level ranged from 22.5 to 28.4 pg/ml with an average and SD value of 24.9 ± 1.7 pg/ml in RA patients below 50 years. RA patients over 50 had a mean ± SD value of 44.5 ± 9.9 pg/ml, ranging from 28.9 to 60.2; (B) The serum levels of TNF-α was statistically significant between males and females (t = 3.378, P = 0.0015).
pg/ml. The difference between males and females was statistically significant \((t = 3.378, P = 0.0015)\), Figure 2B.

**Genotyping of TNF-α 308 promoter polymorphism**

We evaluated the frequency of TNF-308 gene polymorphism in the studied subjects; we found that GG genotypes were more common in RA than in the controls and that GG genotype may be a risk factor to RA and AA genotypes were more frequent in controls than in the RA. The difference in the frequency of TNF-α 308 polymorphism genotypes was statistically significant between RA patients and the controls \((P = 0.0058)\), Table 2.

Analysis of allele’s frequency of TNF-α 308 gene polymorphism revealed that the G allele was more frequent in RA (75%) and in controls (58%), A allele was more in the controls (42%). This difference was statistically significant \(P = 0.0162\), Table 3.

**TNF-α 308 promoter polymorphism and serum level of TNF-α**

The serum level of TNF-α was observed to be elevated in patients with GG genotypes of RA in relation to the controls. The serum levels ranged between 29.02 and 60.15 pg/ml in RA patients with GG genotype with a mean ± SD value of 46.35 ± 8.95 pg/ml. on the other hand, it ranged between 0.24 and 4.7 pg/ml with a mean ± SD values of 2.73 ± 1.3 pg/ml in the controls with low GG genotype. The difference was statistically significant \(t = 21.56, P < 0.0001\), Figure 3.

**Functional disability indices in RA**

There are several indices to evaluate the functional activity of patients with RA, these includes Kellgren score, Lesquene index for activity, and Womac index. We assessed the relation of serum level of TNF-α, CRP, RF and the functional ability of RA patients using the above-mentioned indices. The serum RF and CRP levels were not significantly correlated with different Kellgren scores, Lequesne grades, and Womac index scores. Meanwhile, the TNF-α serum levels were significantly correlated with each grade of Kellgren score \((P < 0.0001)\), with Lequesne index \((P < 0.0001)\), and Womac index \((P < 0.0001)\), Table 4.

**Discussion**

Rheumatoid arthritis (RA) is considered the most prevalent inflammatory arthropathy that results in progressing articular injury, impairment of function and comorbidity. Cytokine plays an important part in pathogenesis of RA. It initiates the inflammatory response that results in edematous joint and subsequent bone destruction during the development of disease [25]. TNF-α is a multifunctional, pro-inflammatory cytokine

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**Table 2:** Genotypes frequency of TNF-α 308 promoter polymorphism among the studied participants.

<table>
<thead>
<tr>
<th></th>
<th>RA patients (N = 50)</th>
<th>Controls (N = 50)</th>
<th>OR (CI 95%)</th>
<th>(\chi^2)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>No 30 (60)</td>
<td>No 20 (40)</td>
<td>3.6 (1.1-11.8)</td>
<td>10.31</td>
<td>0.0058</td>
</tr>
<tr>
<td>GA</td>
<td>15 (30)</td>
<td>18 (36)</td>
<td>2 (0.57-6.97)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>5 (10)</td>
<td>12 (24)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3:** Alleles frequency of TNF-α 308 gene polymorphism among the studied participants.

<table>
<thead>
<tr>
<th>Groups</th>
<th>RA patients (n = 100)</th>
<th>Controls (n = 100)</th>
<th>OR (CI 95%)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (%)</td>
<td>No (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>75 (75)</td>
<td>58 (58)</td>
<td>2.172</td>
<td>0.162</td>
</tr>
<tr>
<td></td>
<td>CI: 1.196 to 3.919</td>
<td></td>
<td></td>
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</tbody>
</table>

OR: Odds Ratio; CI: Confidence Interval.
that plays a significant part in various autoimmune conditions. The TNF-α gene was recognized as a vital factor for autoimmune and inflammatory disorders [26]. The functions played by TNF-α seem conflicting, and this was due to the genetic polymorphisms in the genes that control their development and effect [27]. The genetic alterations in the TNF-α locus have been reported to be involved in high TNF-α output [28]. This research aims to detect the polymorphism of TNF-α (G-308μ) and to determine its relationship with RA.

In the present study, we examined the serum levels of CRP, and TNF-α in addition to ESR in RA patients and the controls. We noted that there is a significant difference in the serum levels of CRP, and TNF-α among RA patients and the controls, P < 0.0001 and P < 0.0001 respectively. Also, the difference in ESR was statistically significant between patients and controls (P < 0.0001). Our results were also noted by other authors [25,27,29,30]. We analyzed the TNF-α serum levels in RA patients in relation to their age and sex. The TNF-α serum level was significantly higher in RA patients with age above 50 years (P < 0.0001). Female patients were also noted to have a significantly higher TNF-α level than male patients (P = 0.0015).

We assessed 308 G > A polymorphism in the TNF-α gene promoter location in patients with RA and healthy controls. Our results showed that in RA patients the genotype frequency of -308 G > A polymorphism was GG (60%), GA (30%), and AA (10%). In the control group, the genotype frequency of -308 G > A polymorphism was GG (40%), GA (36%), and AA (24%). Statistical analysis showed significant difference in the genotype frequency of this polymorphism between two groups (P = 0.0058). Our results in RA patients indicate significantly lower -308 A allele and higher -308 G allele frequencies relative to controls (P = 0.016). These findings are similar to those recorded for different racial groups, including Japanese, Swedish, Turkish, Hungarian [26,31-33]. This research, along with other published studies, supported the view that TNF-α -308 A allele exerts a protective effect on RA development [34]. In contrast to our results, the studies from Australian Caucasian [35], Spanish [36], Mexican [37], and Chilean [38] showed significantly higher rates of allele A in RA patients compared with control group.

In the current study, we found a significant correlation between the serum level of TNF-α and GG genotypes in RA patients relative to the controls (P < 0.0001). Our results support the results of Nemec, et al. who recorded an elevated TNF-α in the plasma and synovial fluid of joints of RA patients with polymorphisms in the TNF-α promoter region [39]. On the contrary, Pawlik, et al. reported no differences in TNF-α serum levels and genotype distribution and allelic frequencies of -308 G > A TNF-α promoter polymorphism between RA patients and controls [40]. The variability in TNFα expression between individuals indicates the presence of functionally distinct TNF alleles, including 308 promoter polymorphism; that may play a role in RA susceptibility [41]. Mousa, et al. found that a significant increase in TNFα expression was observed in Egyptian patients with RA compared to healthy individuals but this increase in expression was not linked to a certain -238G / A and -376G / A single nucleotide polymorphism [42].

The functional disability in RA patients was also evaluated using Kellgren scores, Lesquene grades, and Womac index scores and the serum levels of CRP, RF, and TNF-α. There was no statistically significant correlation between the serum levels of CRP, and RF and the degree of functional disability in RA patients. Meanwhile, the TNF-α serum levels were significantly correlated with the grade or the severity of functional disability in

<table>
<thead>
<tr>
<th>Patients</th>
<th>TNF-α Level</th>
<th>CRP Level</th>
<th>RF Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kelleqern score:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>3 6</td>
<td>17.59 11.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Grade II</td>
<td>22 44</td>
<td>42 10.83</td>
<td>30.39 11.88</td>
</tr>
<tr>
<td>Grade III</td>
<td>7 14</td>
<td>50.51 18.88</td>
<td>31.89 9.288</td>
</tr>
<tr>
<td>Lesquene index for activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild 1-4</td>
<td>16 32</td>
<td>23.45 6.015</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Moderate 5-7</td>
<td>17 34</td>
<td>34.12 9.645</td>
<td>27.61 11.31</td>
</tr>
<tr>
<td>Severe 8-10</td>
<td>4 8</td>
<td>38.26 19.77</td>
<td>42.3 6.053</td>
</tr>
<tr>
<td>Very severe 11-13</td>
<td>9 18</td>
<td>48.15 15.64</td>
<td>29.96 11.61</td>
</tr>
<tr>
<td>Extremely severe ≥ 14</td>
<td>4 8</td>
<td>47.79 23.94</td>
<td>32.52 6.095</td>
</tr>
<tr>
<td>Womac OA index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less severe ≤ median (25)</td>
<td>20 40</td>
<td>25.78 2.457</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>More severe &gt; median (25)</td>
<td>30 60</td>
<td>46.46 8.739</td>
<td>30.2 11.37</td>
</tr>
</tbody>
</table>

Table 4: Correlation of the serum levels of TNF-α, CRP, and RF levels and functional disability indices.
RA patients. Its level was correlated with the grade of disability using the Kellgren score (P < 0.0001), level of disability by Lesquene index (P < 0.0001), and severity of disability by Womac index (P < 0.0001). This observation reveals that the serum level of TNF-α may be used as a measure of functional disability in RA patients.

Conclusion

The present study showed that TNF-α promoter 308 polymorphism GG genotype may be considered as a risk factor for Rheumatoid Arthritis and the TNF-α serum level was significantly related to the functional disability in the disease. However, a future study with a large number of patients is required to emphasize these results.

Declarations Section

Ethical approval and consent to participate

The study was approved by the Committee of Ethics of research, Taif University. Informed consent was obtained from all participating patients before enrollment in the study.

Consent for publication

All authors consent to the publication of the manuscript in ACR, should the article be accepted by the Editor-in-chief upon completion of the refereeing process.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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