Tumor Necrosis Factor Alpha Gene Polymorphism and Association With Its Serum Level in Iranian Population With Rheumatoid Arthritis

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ABSTRACT

Objectives: This study aims to determine whether promoter -238 G/A polymorphism in tumor necrosis factor-alpha (TNF-α) gene is associated with susceptibility to rheumatoid arthritis (RA) in Iranian population and serum level of TNF-α.

Patients and methods: This case-controlled study was performed on two groups including 90 RA patients (20 males, 70 females; mean age 50.3 years; range 26 to 65 years) and 90 healthy controls (21 males, 69 females; mean age 48.6 years; range 27 to 63 years). We determined the frequency of -238 G/A TNF-α gene polymorphism by polymerase chain reaction restriction fragment length polymorphism. We measured the serum level of TNF-α using enzyme-linked immunosorbent assay method. Also, we determined the association of serum TNF-α level with the polymorphism in RA patients.

Results: There was no significant difference in terms of sex and age in the two groups. In the RA group, the genotype frequency of -238 G/A polymorphism was GG (76.6%), GA (17.8%), and AA (5.6%). In the control group, the genotype frequency of -238 G/A polymorphism was GG (83.5%), GA (8.8%), and AA (7.7%). Statistical analysis showed no significant difference in the genotype frequency of this polymorphism between two groups (p=0.07). The serum level of TNF-α were 5.21±1.69 Pg/mL (range 0 to 11.6) in the control group and 62.4±27.1 Pg/mL (range 0 to 117.22) in the RA group (p<0.0001). There was no significant difference in terms of serum TNF-α level and different genotypes in the RA group (p=0.5).

Conclusion: Our findings demonstrate that the TNF-α -238 G/A gene polymorphism may not represent a significant risk factor for RA in Iranian population and there is no association between the polymorphism and serum TNF-α level in RA patients.

Keywords: Polymorphism; rheumatoid arthritis; tumor necrosis factor alpha.

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology that can cause serious weakness and inability. RA is characterized by chronic synovitis, systemic inflammation and the presence of autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (Anti-CCP). This disorder is three times more frequent in females than males. Prevalence of disease increases with age and is highest in females older than 65 years. It is estimated that the disease affects 0.5% to 1.0% of adults in developed countries. A study in the southeastern of Iran showed that the prevalence of RA in the population was 0.98%. Despite several factors which may cause the disease, there is evidence that genetic factors also play an important role and it is estimated that 50% of the risk of developing RA is attributable to genetic factors. 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. 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.\[^8\] TNF-α is produced mostly by activating macrophages although it can be produced by T CD4\(^+\) cells and natural killer cells. It binds to one of its receptor, TNF-receptor type I (p55) or TNF-receptor type II (p75). The TNF-receptor type I is expressed in most tissues and the TNF-receptor type II on immune system cells.\[^9\] The interaction of TNF-α with TNF-receptor type I is a migratory stimulus for T CD4\(^+\) cells in RA subjects.\[^10\] Therefore, this cytokine is necessary for TCD4+ migration in arthritic joints. Elevated serum level of TNF-α has been reported in RA patients.\[^11,12\]

In human, the TNF-α gene is located on the short arm of chromosome 6 in the 6P21 region where the class III region of the human leukocyte antigen genes is located.\[^13\] Several single nucleotide polymorphisms (SNP) have been demonstrated in the promoter region of the TNF-α gene and some of them are known to affect the level of gene expression.\[^14\] Of these, mutation in the promoter region with a G→A substitution in position -238 (rs361525G/A), has been the subject of intense studies.\[^15-17\] A study showed that -238 allele is associated with higher TNF-α production with respect to the -238 G allele,\[^18\] however, this result was not confirmed by other studies.\[^19,20\] The G→A substitution in position -238 in the TNF-α gene is associated with lower production of TNF-α in patients with ulcerative colitis.\[^21\]

To the best of our knowledge, no studies have investigated the potential association between -238 G/A TNF-α gene polymorphism with RA in Iranian population. Therefore, in this study, we aimed to determine whether promoter -238 G/A polymorphism in TNF-α gene is associated with susceptibility to RA in Iranian population and serum level of TNF-α.

PATIENTS AND METHODS

This case-controlled study was performed between March 2012 and December 2013 on two groups including 90 RA patients (20 males, 70 females; mean age 50.3 years; range 26 to 65 years) who referred to the Rheumatology Clinic of Shahid Sadoughi hospital, Yazd, Iran and 90 healthy controls (21 males, 69 females; mean age 48.6 years; range 27 to 63 years) who were randomly selected from the general population who lived in the same area, strictly unrelated, and without history of chronic inflammatory or autoimmune disease. The subjects fulfilled the 2010 American College of Rheumatology-European League Against Rheumatism classification criteria for RA.\[^22\] Disease activity was assessed using the Disease Activity Score 28 (DAS28). Subjects in both groups were matched in terms of age and sex. All patients were examined by a rheumatologist. The Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran approved the study design. Signed informed consent was obtained from each subject. The study was conducted in accordance with the principles of the Declaration of Helsinki.

A sample of peripheral venous blood (10 mL) was taken from all participants. Five milliliters of each blood sample were collected in ethylenediaminetetraacetic acid coated tubes for deoxyribonucleic acid (DNA) extraction and the rest centrifuged after clotting and the serum harvested and stored in -70 °C until used. In the RA group, hemoglobin level was measured by an automated cell counter (CelltacE, Nihon Kohden, Japan). The Westergren method was used to determine the erythrocyte sedimentation rate at the laboratory.

The RF and C-reactive protein (CRP) levels in all sera were measured by standard kit according to manufacturer’s instructions (Pishtaz Teb and Bionic Company, Iran respectively) and using auto analyzer (Prestige 24i, Japan).

Serum concentration of Anti-CCP in the RA group was determined by enzyme-linked immunosorbent assay method according to the manufacturer’s instructions (AESKU. Diagnostics GmbH & Co. KG, Wendelsheim, Germany).

The levels of TNF-α in the RA and control groups were measured by enzyme-linked immunosorbent assay method according to the kit instructions (eBioscience, Bender Med Systems, GmbH, Austria). The sensitivity of TNF-α kit was 1.65 Pg/mL.

Genomic DNA was extracted from peripheral blood using a DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer’s
instructions. Determining of -238 G/A TNF-α was performed using polymerase chain reaction (PCR) restriction fragment length polymorphism method. PCR reaction was carried out in 25 μL reaction volumes containing 100 ng of genomic DNA, 1.5 mM MgCl2, 0.2 mM dNTP, 10 pmol of each primer and 1 unit of Taq DNA polymerase. PCR reagents were purchased from the CinnaGen Company (Tehran, Iran). Primers were designed according to published sequences in the Genome Database (GenBank) using the software PRIMER3. Information on primer sequences is as follows:

Forward: 5'-AGAAGACCCCCCTCGGACTC-3',
Reverse: 5'-GCTGGTCCTCTGCTGTCCTT-3'

The PCR cycling conditions were as follows: five minutes at 94 °C, 30 cycles of denaturation for one minute at 94 °C, annealing for one minute at 61 °C and extension for one minute at 72 °C. A final extension time of five minutes at 72 °C was included at the end of last cycle (Applied Biosystems, ABI, Foster City, CA, USA). The product size of PCR was 315 base pairs (bp) that was confirmed by electrophoresis in a 1% agarose gel stained with the DNA green viewer (Pars-Tous, Iran). The PCR product was digested by an addition restriction enzyme Ddel (Promega, USA, Cat No. R6295) at 37 °C for 60 minutes in a final volume of 20 μL under the following conditions: 10 μL PCR product, 7.3 μL deionized water, 2 μL 10x buffer, 0.5 μL Ddel and 0.2 μL acetylated bovine serum albumin. The resulting fragments of the restriction fragment length polymorphism were analyzed using electrophoresis on 2% gel agarose. All the gels were imaged using E-gel imager (Life Technologies) instrument. Ten samples from each group were randomly selected to perform the repeated assays, and the results were 100% concordant.

**Statistical analysis**

The statistical analyses were performed with the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). SNP were evaluated for deviation from Hardy-Weinberg Equilibrium for both RA and control groups by a Chi-square test. The genotype and allele frequencies of -238 G/A polymorphism were calculated by direct count. Chi-square test was used to evaluate the differences between the genotype frequency in the RA and control groups. The mean of serum TNF-α between RA and control groups and comparison mean serum concentrations of TNF-α in the RA and control groups in the genotypes GG, GA and AA were analyzed with Mann-Whitney U test. In RA group, Spearman’s correlation test was used to test the correlation between the mean serum TNF-α level and anti-CCP, RF, and CRP. The data for serum TNF-α are shown as mean ± standard error of mean and for the others as mean ± standard deviation. P-values less than 0.05 were considered statistically significant.

**RESULTS**

All participants of the study (90 RA patients and 90 controls) were genotyped for the -238G/A TNF-α gene polymorphism. The TNF-α genotype frequencies were in Hardy-Weinberg Equilibrium in two groups (p>0.05). Demographic and clinical characteristics of subjects are summarized in

| Table 1. Main demographic and clinical characteristics of rheumatoid factor patients and controls |
|--------------------------------------------------|--------------------------------------------------|
| **RA patients (n=90)** | **Controls (n=90)** |
| **Gender** | **%** | **Mean±SD** | **Range** | **%** | **Mean±SD** | **Range** |
| Female | 77.8 | 50.3±9.1 | 26-65 | 76.6 | 48.6±12.4 | 27-63 |
| Male | 22.2 | 23.4 | | | | |
| **Age (year)** | | | | | | |
| Age at diagnosis (year) | 43.8±10.2 | - | | | | |
| Duration of the disease (year) | 5.1±2.93 | - | | | | |
| Disease Activity Score 28 | 3.6±0.8 | - | | | | |
| Hemoglobin levels (g/dL) | 12.94±1.31 | - | | | | |
| Erythrocyte sedimentation rate | 28.48±19.23 | - | | | | |
| Rheumatoid factor (lu/mL) | 15.42±8.69 | 9.11±2.33 | | | | |
| C-reactive protein (mg/dL) | 17.84±11.48 | 4.05±2.04 | | | | |
| Anti-cyclic citrullinated peptide (U/mL) | 189.57±127.96 | - | | | | |

RA: Rheumatoid factor; SD: Standard deviation.
Table 1. There was no significant difference between the groups in terms of sex and age. Levels of RF were significantly different between the groups (p<0.001). Also, RA patients had a significantly higher level of CRP than the controls (p<0.001). Serum level of TNF-\( \alpha \) was 5.21±1.69 Pg/mL (range 0 to 11.6) in the control group and 62.4±27.1 Pg/mL (range 0 to 117.22) in the RA group. RA patients showed significantly increased serum TNF-\( \alpha \) level as compared to controls (p<0.0001). In the RA group, there was no correlation between TNF-\( \alpha \) serum concentration and serum levels of anti-CCP (r=0.05, p=0.5), RF (r=0.09, p=0.3), and CRP (r=0.12, p=0.2).

The product size was 315 bp for SNP -238 G/A (Figure 1). There were three bands (262, 24 and 13 bp) in the presence of homozygous AA and two bands (275 and 24 bp) in the presence of homozygous GG after enzymatic digestion by Ddel. GG genotype was the most frequent allele in RA (76.6%) and control groups (83.5%). There was no significant difference in the genotype frequencies of the -238 G/A polymorphism between both groups (p=0.07). Also, G allele was the dominant allele in both the RA (85.5%) and control (87.2%) groups. The allele frequencies of the -238 G/A polymorphism between two groups were similar (p=0.1). The data are shown in Table 2. The mean serum levels of TNF-\( \alpha \) in RA patients with different genotypes of -238 G/A were compared. Our results showed that no significant differences in serum TNF-\( \alpha \) levels and different genotypes (p=0.5). Mean of DAS28 in GG, GA, and AA genotypes were 3.3±0.9, 3.1±0.6, and 4.5±1.1, respectively. AA genotype showed a statistically significant higher DAS28 than GG and GA genotypes (p=0.01).

**DISCUSSION**

Cytokines are important mediators of the immune system and imbalance or deficiency in the cytokine network may lead to autoimmunity disease susceptibility. TNF-\( \alpha \) is a multifunctional, proinflammatory cytokine, which plays an important role in many autoimmune diseases. The TNF-\( \alpha \) gene has been postulated as a key genetic factor in inflammatory and autoimmune diseases.23 The gene is tightly regulated at the level of transcription.24,25 In this study, we compared the serum level of TNF-\( \alpha \) in RA patients and healthy controls. Our results showed that RA patients had a significantly higher level of TNF-\( \alpha \) than controls. TNF-\( \alpha \) has an important role in the regulation of a cascade of pathogenesis events leading to RA. This cytokine is produced in high concentration by a number of cells, such as monocyte and T cells in RA.26 The pivotal role of TNF-\( \alpha \) in pathogenesis of RA has been demonstrated in successful clinical trials targeting TNF-\( \alpha \) and in collagen-induced arthritis in mice as a model of RA.27 Other authors reported elevation of serum TNF-\( \alpha \) in RA patients and our results are consistent with previous findings.11,28,29

In our study, there was no correlation between serum TNF-\( \alpha \) concentration and the serum levels of RF, anti-CCP, erythrocyte sedimentation rate,

![Figure 1. Gel picture showing polymerase chain reaction product for -238 GA (rs361525). The polymerase chain reaction product size was 315 bp.](image)

<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele frequency</th>
<th>Genotype frequency</th>
</tr>
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<tbody>
<tr>
<td>rs361525G/A</td>
<td>G Allele</td>
<td>A Allele</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Rheumatoid arthritis patients</td>
<td>154</td>
<td>85.5*</td>
</tr>
<tr>
<td>Control group</td>
<td>158</td>
<td>87.8</td>
</tr>
</tbody>
</table>

SNP: Single nucleotide polymorphisms; * p=0.1; **p=0.07.
and CRP in RA patients. RF is detectable in nearly 70% of RA patients, although its presence is not specific for RA.\(^{30}\) Also, anti-CCP assays are detectable in about 70% of patients with RA and are highly specific for RA.\(^{31}\) CRP is an acute-phase protein mostly synthesized by hepatocytes and Kupffer cells in the liver and is regulated by cytokines such as TNF-\(\alpha\) and IL-6.\(^{32}\) As expected, the concentration of CRP was significantly increased in RA patients as compared to the controls. In contrast to our results, the correlation of TNF-\(\alpha\) level with RF, CRP, and erythrocyte sedimentation rate was reported in RA patients.\(^{33}\)

We determined \(-238\) G/A polymorphism in the promoter region of the TNF-\(\alpha\) gene in RA patients and healthy controls. Our findings showed that genotype GG in RA (76.6%) and control groups (83.5%) had the highest frequency. The GA genotype frequency was 17.8% in RA group and 8.8% in the control group. The frequency of the AA genotype in both RA and control groups had the lowest frequency. This SNP polymorphism was studied in some disorders in different countries. Zammiti et al.\(^{34}\) studied association between TNF-\(\alpha\) polymorphism and idiopathic recurrent miscarriage in Tunisia population. The GG genotype frequency was 71.1% in the cases and 81% in the controls and the AA genotype had the lowest frequency in both groups. This polymorphism was surveyed in Turkish RA patients and compared with control subjects. The results showed that the GG genotype frequency was 96% in both case and control groups and the AA genotype was not found in the two groups.\(^{35}\) The \(-238\) G/A polymorphism in TNF-\(\alpha\) gene was determined in Chinese patients with Graves’ disease by Gu et al.\(^{36}\) where the GG genotype frequency was 95.3% in patients and 89.5% in the controls and the AA genotype was not detectable in the two groups. Also, the frequency of GG genotype was 88.65% in tuberculosis patients and 90.97% in controls in Asian Indians.\(^{37}\) The association of this polymorphism with Paget’s disease of bone was studied in the Spanish population. The results showed that GG genotype in patients (88%) and controls (86%) had the highest frequency.\(^{38}\) The frequency of genotypes in these studies is consistent with our results but the prevalence of the genotypes is different. The difference in race, sample size, and sampling might explain this discrepancy.

We could not find a significant difference in the genotype frequencies of the \(-238\) G/A polymorphism between the RA and control groups. In a study, researchers investigated TNF-\(\alpha\) \(-238\) polymorphism and association with messenger ribonucleic acid expression and soluble TNF-\(\alpha\) in 50 RA patients and 100 healthy subjects. No significant difference in TNF-\(\alpha\) \(-238\) G/A polymorphism was observed in both groups.\(^{33}\) Fabris et al.\(^{39}\) investigated this polymorphism in 100 RA patients with severe and mild disease and 45 healthy blood donors. They reported that AG genotype was absent in severe-unresponsive RA, but present in mild-responsive RA patients and GG homozygosity associated with severity and unresponsiveness. In a prospective study, the correlation between TNF-\(\alpha\) \(-238\)G/A promoter polymorphism with joint damage in RA was determined. The results showed that TNF-\(\alpha\) GG genotype contributed to the progression of joint destruction in RA.\(^{40}\) Association of \(-238\) G/A polymorphism with pathogenesis in some autoimmune diseases was studied. The results of various studies on this topic have been inconsistent. Laddha et al.\(^{41}\) reported significant association of this SNP with generalized and localized vitiligo. The study performed on Chinese patients with Graves’ disease indicated that the distribution of \(-238\) G/A genotypic and allelic frequencies between patient and control subjects was significantly different. The G allele of TNF-\(\alpha\) \(-238\) conferred a higher risk of Graves’ disease as compared with A allele.\(^{36}\) Ferguson et al.\(^{42}\) studied association of this SNP in the TNF-\(\alpha\) gene with inflammatory bowel disease risk. They did not find a significant difference between controls and Crohn’s disease patients, and ulcerative colitis patients and controls. Independent association of TNF-\(\alpha\) polymorphism with type 1 diabetes susceptibility has been reported.\(^{43}\)

As part of our research, we compared serum TNF-\(\alpha\) level in the different genotypes in RA patients and found that the serum TNF-\(\alpha\) levels were similar in different genotypes. Our result is not compatible with a study conducted on RA patients, which showed that GG genotype was associated with higher serum TNF levels than GA genotype.\(^{33}\) Also, another study on vitiligo patients showed that patients with GA and AA
genotype had a significant increase in serum TNF-α level as compared to controls.\textsuperscript{41}

We found a significant difference between genotypes and DAS28 in RA patients. The AA genotype had the highest DAS28 value. The difference in DAS activity score between genotypes may predict the treatment outcome in RA patients.

There are some limitations to our study, which could affect the results. There were no consideration and analysis of the type of drugs used by the patients. Some drugs can affect the serum TNF-α level. In addition, one SNP was determined and compared between RA patients and controls. Furthermore, our sample size may not be large enough which may have weakened our ability for a reliable statistical association.

In conclusion, the findings of the present study indicate that -238G/A polymorphism in TNF-α gene might not be associated with a high risk of RA in Iranian patients and this SNP do not affect the serum level of TNF-α in RA patients. Further prospective studies on larger sample sizes with different ethnicities are required to confirm our findings. Also, multiple SNPs should be considered in future studies.

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