

# Tumor Necrosis Factor Gene Polymorphisms in Advanced Non-exudative Age-related Macular Degeneration

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## Abstract

**Purpose:** To investigate tumor necrosis factor (TNF)- $\alpha$  gene polymorphisms in advanced dry-type age-related macular degeneration (AMD) in a population from Northeastern Iran.

**Methods:** In this case-control study, 50 patients with geographic macular atrophy and 73 gender-matched controls were enrolled. Genomic deoxyribonucleic acid (DNA) was extracted from the peripheral blood. Polymerase chain reaction was performed to analyze 2 candidate single nucleotide polymorphisms in the TNF- $\alpha$  gene, namely -1031 thymine (T)/cytosine (C) and -308 guanine (G)/adenine (A).

**Results:** The distribution of the -1031 T/C genotype was TT, 62%; TC, 36%; CC, 2% in the patients and TT, 60%; TC, 36%; CC, 4% in the controls ( $P = 0.94$ ). Genotype analysis of TNF- $\alpha$  -308 also revealed no significant difference in distribution between patients (G, 78%; GA, 22%; AA, 0%) and controls (GG, 74%; GA, 23%; AA, 3%) ( $P = 0.51$ ). None of the haplotypes nor alleles of studied TNF- $\alpha$  polymorphisms were significantly associated with advanced dry-type AMD.

**Conclusion:** The findings of this study show that polymorphisms in the TNF- $\alpha$  gene, do not play an important role in dry-type AMD in the studied population.

**Keywords:** Age-related Macular Degeneration; Gene Polymorphism; Geographic Atrophy; Tumor Necrosis Factor- $\alpha$

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## INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of central visual loss and irreversible blindness

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in aging patients, resulting in a tremendous decrease in quality of life in the geriatric population worldwide. Whereas the increase in life expectancy has become more noticeable, it becomes as one of the leading health problems.<sup>[1-4]</sup> Inflammation has been suggested to play a major role in the pathogenesis of this complex and multifactorial disease.<sup>[5-7]</sup>

Since the discovery of the relationship between genetic Polymorphisms and AMD, knowledge of the genetic

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basis of this disease has advanced significantly.<sup>[8-11]</sup> Evaluation of genetic polymorphisms can lead to major developments in the field of AMD through modifications in preventive and therapeutic approaches, according to the special genetic profile of each patient.

As a pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF- $\alpha$ ) plays a pivotal role in regulation of the immune response. The role of TNF- $\alpha$  gene polymorphisms in AMD and anti-TNF- $\alpha$  treatment for AMD, has been investigated.<sup>[12,13]</sup> Several single nucleotide polymorphisms (SNPs) in the TNF- $\alpha$  promoter region have been identified from which the -1031C and -308G alleles have been shown to enhance TNF- $\alpha$  production level,<sup>[14,15]</sup> indicating the possible role of these genetic polymorphisms on pathologic processes involving TNF.

Gonabad is an ancient Northeastern city in Iran and has one of the longest life expectancies in Iran (based on the 2007 National Census). To better understand the role of TNF- $\alpha$  gene polymorphisms, this study investigated the prevalence of TNF- $\alpha$  gene polymorphisms in patients with geographic macular atrophy compared with controls in this population. To the best of the authors' knowledge, this is the first study of TNF- $\alpha$  genetic polymorphisms in Iranian AMD patients.

## METHODS

This prospective, hospital-based case-control study was approved by the Review Board/Ethics Committee of Gonabad University. The study protocol was explained to all patients and informed consent was obtained. We evaluated patients who were sixty years of age or older at Gonabad Hospital from 2011 to 2013. All enrolled patients had advanced dry-type AMD (geographic atrophy) in at least one eye. Geographic atrophy (GA) was defined as an area of retinal pigment epithelial depigmentation occurring in the macula (defined as a 3000-micron diameter circle centered at the fovea) within which underlying choroidal blood vessels are usually visualized. All patients underwent a precise ophthalmologic examination and imaging by two ophthalmologists to confirm the diagnosis and exclude other possible etiologies, which could result in similar macular pathology. Color fundus photography and fluorescein angiography were performed for all enrolled subjects.

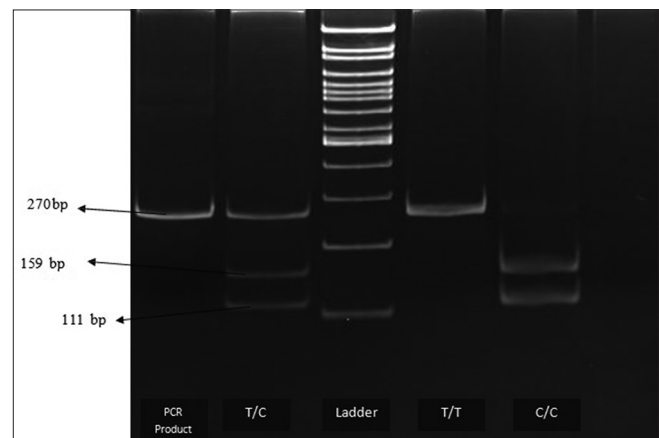
The control group was randomly selected from the outpatient general clinic during routine examination. They were ethnically-and sex-matched with the study group and there was no visual impairment. They underwent dilated fundus examination to confirm the absence of any sign of AMD or retinal disorders. They did not have family history of AMD in their first-degree relatives. Individuals with severe cataracts impairing

visualization of the macula were excluded. All patients and healthy control subjects were unrelated to each other and had no other retinal, renal, cardiovascular, hematologic disease or diabetes mellitus.

The detailed method of deoxyribonucleic acid (DNA) extraction and polymerase chain reaction assay has been described in a previous study.<sup>[16]</sup> Genomic DNA was extracted from peripheral blood cells using the simple salting out procedure.<sup>[17]</sup> Polymorphisms in the promoter region of the TNF- $\alpha$  gene at positions -1031 thymine (T)/cytosine (C) and -308 guanine (G)/adenine (A) were detected by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The 270-bp region of the TNF- $\alpha$  gene, encompassing the -1031T/C polymorphism site and the 107-bp region encompassing the -308 G/A polymorphism site were amplified by PCR.<sup>[16]</sup> Agarose gel electrophoresis was used for verification of the PCR products and digested PCR products were electrophoresed in polyacrylamide gel and visualized by ethidium bromide staining [Figures 1 and 2].

## Statistical Analysis

To determine whether each SNP was on the Hardy-Weinberg equilibrium (HWE), the observed genotype frequency distributions were compared with the expected ones, using the Chi-square test. Comparison of alleles and genotype frequencies between patients and healthy controls was performed using the Chi-square test or Fisher's exact test, where appropriate. Differences in haplotype frequencies of the TNF- $\alpha$  promoter region between patients and healthy controls were also analyzed using the Chi-square test. Unconditional logistic regression was used after adjusting for age. Probability values of less than 0.05 were regarded as statistically significant.

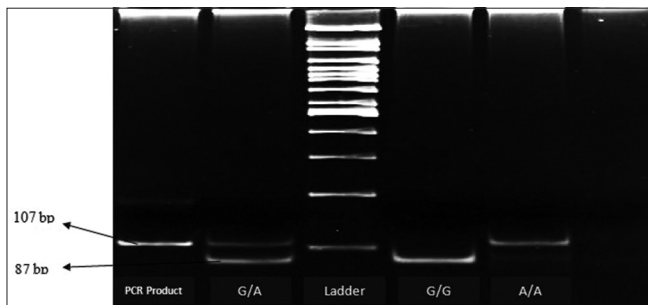


**Figure 1.** Agarose gel electrophoresis for verification of the polymerase chain reaction (PCR) products and polyacrylamide gel electrophoresis for their separation (TNF- $\alpha$ -1031 T/C). T, thymine; C, cytosine.

**RESULTS**

Baseline characteristics of patients with geographic atrophy and controls are presented in Table 1. There was no significant difference in age, sex or cigarette smoking in the study group as compared to controls [Table 1]. It was confirmed that the genotypic frequencies, both in patients and controls, fit the HWE for both TNF- $\alpha$  gene polymorphisms.

The frequency of the -1031 T/C genotype distribution was TT, 62%; TC, 36%; CC, 2% in patients and TT, 60%; TC, 36%; CC, 4% in the control group ( $P = 0.94$ ). In a similar fashion, genotype analysis of TNF- $\alpha$  -308 also revealed no significant difference in distribution ( $P = 0.51$ ) between patients (GG, 78%; GA, 22%; AA, 0%) and controls (GG, 74%; GA, 23%; AA, 3%). None of the alleles of the studied TNF- $\alpha$  polymorphisms were significantly associated with geographic atrophy [Table 2]. Although the CA haplotype was not observed in any of the patients, none of the haplotypes had a significantly higher frequency in patients with geographic atrophy [Table 3]. These analyses were repeated using logistic regression, adjusting for age and none of the genotypes, alleles or haplotypes were significantly associated with geographic atrophy.



**Figure 2.** Agarose gel electrophoresis for verification of the polymerase chain reaction (PCR) products and polyacrylamide gel electrophoresis for their separation (TNF- $\alpha$ : -308 G/A). G, guanine; A, adenine.

**DISCUSSION**

This prospective, hospital-based, case-control study showed that two candidate SNPs of the TNF- $\alpha$  gene polymorphisms at -1031T/C and -308G/A, did not demonstrate a significant association with advanced dry-type AMD, in this population from Northeastern Iran. Genetic knowledge on AMD has expanded tremendously and the role of inflammation in the development of AMD has become evident.<sup>[5-7]</sup> The pro-inflammatory TNF- $\alpha$  is a multifunctional cytokine which plays a pivotal role in the regulation of the immune response. It is located on chromosome 6p21.3 and consists of 4 exons and 3 introns. Systemic<sup>[18]</sup> and intravitreal<sup>[12]</sup> anti-TNF- $\alpha$  agents such as infliximab have shown a remarkable therapeutic effect in wet-type AMD confirming the important role of TNF- $\alpha$  in wet AMD.

In this study which originated from a genetically pure population from Northeastern Iran (Gonabad City), polymorphisms in -1031C and -308G alleles of the TNF- $\alpha$  promoter were studied. The polymorphisms have been found to be located within the promoter non-coding region of the gene and shown to enhance TNF- $\alpha$  production.<sup>[14,15]</sup>

**Table 1. Baseline characteristics of the patients**

|                               | Control group (n=73) | Geographic atrophy (n=50) | P    |
|-------------------------------|----------------------|---------------------------|------|
| Sex <sup>†</sup>              |                      |                           |      |
| Male/female                   | 37/36                | 25/25                     | 0.94 |
| Smoking <sup>†</sup>          |                      |                           |      |
| Smoker/nonsmoker              | 7/66                 | 5/45                      | 0.94 |
| Age (mean $\pm$ SD)*          | 72.42 $\pm$ 7.45     | 75.88 $\pm$ 7.20          | 0.01 |
| Age distribution <sup>†</sup> |                      |                           |      |
| 60-69 year                    | 26                   | 11                        | 0.06 |
| 70-79 year                    | 35                   | 22                        |      |
| >80 year                      | 12                   | 17                        |      |

Values are mean $\pm$ SD. \*Student's *t*-test; <sup>†</sup>Chi-square test of independence. SD, standard deviation

**Table 2. TNF- $\alpha$  polymorphisms in Northeastern Iranian patients with advanced dry-type AMD versus healthy controls**

| SNP      | Genotype frequency (%) |            |            | P            | Allele frequency (%) |          | P            |
|----------|------------------------|------------|------------|--------------|----------------------|----------|--------------|
|          | T/T                    | T/C        | C/C        |              | T                    | C        |              |
| -1031T/C |                        |            |            |              |                      |          |              |
| Patients | 31 (62)                | 18 (36)    | 1 (2)      | 0.94 (0.81*) | 80 (80)              | 20 (20)  | 0.86 (0.72*) |
| Controls | 44 (60)                | 26 (36)    | 3 (4)      |              | 114 (78)             | 32 (22)  |              |
|          | <b>G/G</b>             | <b>G/A</b> | <b>A/A</b> |              | <b>G</b>             | <b>A</b> |              |
| -308G/A  |                        |            |            |              |                      |          |              |
| Patients | 38 (78)                | 11 (22)    | 0 (0)      | 0.51 (0.50*) | 87 (89)              | 11 (11)  | 0.67 (0.47*) |
| Controls | 54 (74)                | 17 (23)    | 2 (3)      |              | 125 (86)             | 21 (14)  |              |

\*Age adjusted *P* values. T, thymine; C, cytosine; G, guanine; A, adenine; TNF- $\alpha$ , tumor necrosis factor alpha; AMD, age-related macular degeneration; SNP, single nucleotide polymorphism

**Table 3. Haplotype frequency of TNF- $\alpha$  promoter region in patients with advanced dry type AMD versus healthy controls**

| Haplotype | Patients | Controls | P            |
|-----------|----------|----------|--------------|
| GT        | 67       | 94       | 0.24 (0.67*) |
| AT        | 7        | 17       | 0.29 (0.23*) |
| GC        | 16       | 28       | 0.68 (0.52*) |
| AC        | 0        | 1        | 0.60 (0.40*) |

\*Age-adjusted P values. T, thymine; C, cytosine; G, guanine; A, adenine; AMD, age-related macular degeneration; TNF, tumor necrosis factor

Only patients with the geographic atrophy variant of dry-type AMD were selected for the purpose of this study. As a result of extensive ethnic migration in Iran, few ophthalmology centers in Iran have access to genetically pure populations with AMD and genetic purity of such studies could not be confirmed. Our prospective study was designed in the specified region of Iran, and the results could be regarded representative of Northeastern Iran. According to the 2007 national census, approximately 10% of the population in this region is aged more than sixty. Approximately 30% of the population over 60 years of age in this region was examined and all eligible cases were included (50 patients). Meanwhile, normal subjects were randomly included from the same population to serve as the control group.

Although neovascular AMD is responsible for the majority of cases of severe visual loss in AMD, geographic atrophy is also regarded as a significant cause of vision loss. There are many treatment modalities, although partially effective, for neovascular AMD, but there is no proven therapy for atrophic disease. The attempt to clarify its pathogenesis is an important step toward developing effective therapies for this form of AMD.

Induction of retinal neovascularization by TNF- $\alpha$  in experimental animal studies has already been demonstrated.<sup>[19]</sup> Moreover, the effect of anti-TNF- $\alpha$  agents in regression of choroidal neovascularization has also been demonstrated.<sup>[12,18]</sup> In another study important role of one of TNF family agents via different levels of its receptors have been shown in AMD.<sup>[20]</sup> Controversially, there was no statistically significant association between systemic TNF levels and early or late AMD in another multi-ethnic study.<sup>[21]</sup> The role of TNF- $\alpha$  gene polymorphisms in wet-type AMD has recently been investigated in Chinese patients from Taiwan.<sup>[13]</sup> These factors suggest the important role of the TNF- $\alpha$  gene in wet-type AMD. The role of TNF- $\alpha$  gene polymorphisms in geographic atrophy susceptibility has not been investigated before.

In this case-control study, two candidate SNPs of the TNF- $\alpha$  gene were investigated to determine the possible association of its polymorphisms with geographic atrophy. Neither of the studied genotypes or alleles of TNF- $\alpha$  gene polymorphisms at TNF $\alpha$  -1031 T/C and

TNF $\alpha$  -308 G/A loci, nor their combined haplotypes were associated with geographic atrophy in this study population.

In contrast to a previous study from Taiwan on Chinese patients with wet AMD,<sup>[13]</sup> the findings of this study does not suggest the role of TNF- $\alpha$  gene polymorphisms in geographic atrophy susceptibility, this difference could be due to ethnic differences or differences in AMD types in these studies. Although some studies have suggested different pathogenic mechanisms for atrophic and wet AMD,<sup>[22]</sup> other studies have revealed that atrophic AMD can transform into wet AMD and vice versa.<sup>[23]</sup> The phenotypical complexity of AMD indicates the possibility of multiple interactions between genetic and environmental factors reflecting the complexity of genetic studies in AMD.

In summary, this study could not find any significant association between TNF- $\alpha$  gene polymorphisms and geographic atrophy. Studies with larger sample size, more SNP candidates and inclusion of multiple ethnic groups are recommended in which the possible role of TNF- $\alpha$  gene polymorphism in the pathogenesis of geographic atrophy may be revealed.

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Nil.

### Conflicts of Interest

There are no conflicts of interest.

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