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Original Article

TNF-alpha 308G/A Polymorphism and Serum Level of TNF-α Associated with COVID-19 Severity

Nahid Askari[1,*] | Sara Shafieipour[2] | Fatemeh Noormand[3] | Elahe Karami Raviz[3] | Soudeh Khanamani Falahatipour[4]

[1]Department of Biotechnology, Institute of Sciences and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran

[2]Gastroenterology and Hepatology Research Center, Institute of Basic and Clinical Physiology Sciences, Kerman University of Medical Sciences, Kerman, Iran

[3]Kerman University of Medical Science, Kerman, Iran

[4]Pistachio Safety Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran

*Corresponding Author E-mail: nahidaskari@gmail.com

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Abstract

Introduction: T-lymphocyte-derived cytokines, including TNF-α, play a critical role in regulating cellular functions, particularly the immune response to pathogenic infections. This study investigated the frequency of TNF-α gene 308 promoter alleles and measured the serum cytokine levels in both control and patient groups.

Methodology: The study was a case-control study that included 84 COVID-19 positive patients from Kerman's Afzalipur Hospital, Iran, and 84 healthy individuals with negative coronavirus test results as the control group. It was used bioinformatics analysis and the PCR-RFLP technique to determine the TNF promoter region's genotype and the ELISA test to measure the cytokine's serum level. Statistical analysis was conducted using SPSS software (Version-20) with a significance level of $P<0.05$.

Results: It was found that the TNF promoter region's 308 SNP genotype was associated with TFII-I transcription factor binding predictions. However, there was no significant difference in allele frequency between the control and case groups in the TNF-308 promoter ($P<0.05$). Patients with COVID-19 had higher serum levels of TNF-α compared to the control group ($P<0.05$). The study also revealed that elevated levels of TNF-α, LDH, ESR, and CRP in the serum can predict severe outcomes in COVID-19.

Conclusion: Although the study did not identify significant differences in TNF-α allele -308 frequencies between COVID-19 patients and the control group, the results suggest that TNF-α alleles may impact the infection severity. As a result, measuring serum TNF-α levels and identifying TNF-α alleles in COVID-19 patients could be valuable for predicting disease severity and developing targeted therapies.

Keywords: Promoter, Bioinformatics analysis, PCR-RFLP allele frequency, Immune response.

Introduction

The outbreak of COVID-19 has become a global health crisis, with millions of cases and deaths worldwide. The disease's clinical manifestations range from mild flu-like symptoms to severe respiratory distress, multi-organ failure, and death. The cytokine storm, a severe immune response caused by an excessive production of pro-inflammatory cytokines, is a crucial factor in COVID-19 pathogenesis [1]. TNF-α is one of the most important cytokines involved in this process and plays a critical role in the host's defense mechanism against a wide range of pathogens. The TNF-α level in the serum or plasma of COVID-19 patients has been found to be associated with disease prognosis. Studies have shown that elevated levels of TNF-α are associated with more severe disease outcomes, such as acute respiratory distress syndrome, multi-organ failure, and death. Therefore, TNF-α has been identified as a possible therapeutic target for COVID-19 treatment [2,5]. Some genes that control inflammation and immunity may have different versions that can affect how the body reacts to various diseases and infections. One of these genes is called TNF-α and it has some changes in the part that controls how much of it is made. By studying how these changes are related to the amount of TNF-α in the blood of people who have COVID-19 and those who do not, we may learn more about how to treat them and help them survive.

This study aims to investigate the TNF-α promoter's genetic polymorphisms and serum levels in COVID-19 patients compared to healthy individuals. The study used a case-control design that includes COVID-19 patients and healthy individuals as the control group.

The TNF-α promoter polymorphisms identified using polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) techniques and confirmed by sequencing. The serum levels of TNF-α measured using the ELISA approach.

The study's findings may have significant implications for COVID-19 management, as they may identify specific genetic factors that contribute to disease susceptibility and severity. This knowledge could be used to develop targeted therapies and personalized treatment strategies for COVID-19 patients. Moreover, understanding the role of TNF-α in COVID-19 pathogenesis may lead to the discovery of new therapeutic targets for the disease. Ultimately, this study may contribute to the global effort to combat the COVID-19 pandemic.

Materials and Method

Promoter analysis and transcription factor binding site prediction: The PROMO version 8.3 database (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) was used to analyze SNP sequences. A query sequence including 1000 bp in FASTA format was used as the query sequence to search for potential binding sites. A maximum matrix dissimilarity rate of 15 was set for prediction using only human transcription factors.

Sample size prediction: The appropriate sample size was calculated using the following Equation (1):

$$N= (z^2p (1-p))/d^2 \quad (1)$$

Where, n is the sample size, Z is the confidence level statistic, P is the predicted prevalence level (obtained from a pilot study or similar research), and d is precision, which corresponds to the effect size. The standard level of confidence was set at 95%.

This hospital-based case-control study included COVID-19 cases (n = 84) and healthy controls (n = 84). Both patients and healthy controls participating in this study had been referred to the hospital for COVID-19 testing in January and February, 2022.

PCR–RFLP and enzyme-linked immunosorbent assay (ELISA): The study was approved by the Research Ethical Committee, Kerman University of Medical Science (with ethical approval no.: IR.KUMS.REC.1400.228 at KUMS). Informed written consent was obtained from all participants after describing the study.

PCR-RFLP and ELISA methods were used to evaluate TNF-α cytokine levels in patients and compare them with controls. A case-control study design was used to estimate the relative risk factors. Blood samples were collected from 84 COVID-19 patients and 84 healthy controls from Afzalipur Hospital in Kerman, Iran. A questionnaire was used to collect information on the patients' names, family members, gender, age, history of COVID-19 vaccine injection, sickness history, date of diagnosis, hospitalization history, and treatment history. Routine blood samples were used to obtain C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), lactate dehydrogenase (LDH), and blood count. TNF-α concentrations were measured in the serum extracted from whole blood using an ELISA kit (Parsgene, Tehran, Iran) following the manufacturer's instructions and read at 450 nm with a microplate reader (BIO-TEK INSTRUMENTS, U.S.A).

To isolate genomic DNA from blood samples, the DENAZIST blood DNA genomic extraction kit (Mashhad, Iran) was used according to the manufacturer's instructions. The isolated samples underwent 1.5% agarose gel electrophoresis to assess the genomic DNA quality. After DNA quality was checked, the DNA quantity was measured using spectrophotometry by measuring the absorbance at 260 nm and 280 nm. After assessing DNA quality, PCR reactions were done in 20 µl reactions using CinnaGen PCR Master Kit according to the protocol by the supplier (CinnaGen., Iran).

The regions surrounding the 308G/A polymorphism in the genome were amplified using the forward primer 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and reverse primer 5′-TCCTCCCTGCTCCGATTCCG-3′. To perform RFLP, the NcoI restriction enzyme was used to digest the 107 bp PCR product following the manufacturer's instructions. Standard protocols for RFLP analysis were then performed on the samples to obtain various bp genotypes. After categorizing the genotypes (wild type, hetero, and homo genotypes), they underwent statistical analysis.

Electrophoresis was conducted to run PCR products on 8% denaturing polyacrylamide gels. PCR products were sized using a DNA ladder (100 bp) as a standard. The PCR products were visualized by staining the gels with silver staining [9] and drying and scanning them so that the genotypes could be scored using UVIDOC software.

The Mann-Whitney U test was used to compare differences between two independent groups. PCR-RFLP data were analyzed by SPSS, version 20.0, and evaluated statistically by Fisher's exact test, with a significance level of $p<0.05$.

The association between TNF-promoter polymorphisms and serum levels in COVID-19 patients and healthy controls was investigated using a hospital-based case-control design in this study. Promoter analysis and transcription factor binding site prediction were conducted using the PROMO version 8.3 database. Sample size prediction was calculated using a

standard formula. PCR-RFLP and ELISA methods were used to evaluate TNF-α cytokine levels, and genomic DNA was isolated from blood samples using a DENAZIST kit. The study was approved by the Research Ethical Committee, and informed written consent was obtained from all participants. The Mann-Whitney U test and Fisher's exact test were used for statistical analysis. The findings of the study could have ramifications for COVID-19 management and the global effort to combat the pandemic.

Results and Discussion

Bioinformatics analysis of TF binding sites on the TNFα promoter: We analyzed 1000-bp DNA sequence promoters in the PROMO database to determine whether there were TF binding sites (Supplementary file-1). The schematic representation of transcription factor binding in Figure 1 shows that the TFII-I transcription factor family was predicted for 308 positions (yellow box).

Figure 1 TF binding predictions in the TNFα promoter associated with 308 SNP genotypes

The study included patients with ages ranging from 25 to 89 years old. Blood pressure was found to be the most prevalent disease in the study group, followed by diabetes, hypothyroidism, respiratory problems, kidney disease, fatty liver, mental problems, and lymphoma, respectively.

The quantity and quality of DNA extracted from blood samples were assessed, and PCR reactions were conducted. The 107 bp fragments were produced by amplifying the genomic region encompassing the 308G/A polymorphism using specific primers. Electrophoresis was performed to run the fragments on 8% denaturing polyacrylamide gels. The products were then sized using a DNA ladder as a standard. Silver staining was applied to the PCR products for visualization. After that, they were dried and scanned for the genotypes to be scored by UVIDOC software.

The PCR product was enzymatically digested by NcoI restriction enzyme and incubated for 12 hours at 37°C to perform RFLP (restriction fragment length polymorphism). The products of the TNF-α (308G/A) PCR-RFLP were visualized on 8% denaturing polyacrylamide gels. The observed bands included 1) two 20 and 87 bp fragments resulting from a complete NcoI cut representing homozygous TNF-α (308G/G), 2) three 20, 87, and 107 bp fragments, resulting from a partial cut representing heterozygous TNF-α (308G/A), and 3) a 107 bp uncut fragment representing homozygous TNF-α (308A/A) (Figure 2).

The GG genotype, the GA genotype, and the AA genotype comprised 81%, 13% and 6% of the 84 patients genotyped for 308G/A, respectively. This was not significantly different from the results in the control group, with the GG, GA, and AA genotypes comprising 82%, 15%, and 3% of the healthy cases.

Although LDH and CRP are valuable tests for the early detection of patients with COVID-19 who require respiratory monitoring, ESR, CRP, and LDH are non-specific biochemical markers. ESR is a general sign of inflammation, while a high ESR indicates body inflammation.

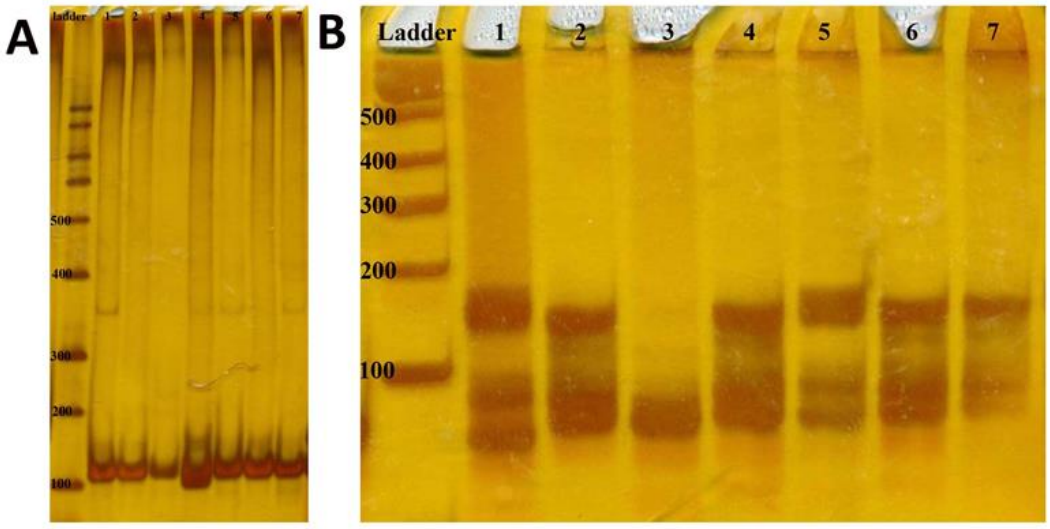

Figure 2 Polyacrylamide (8%) gel electrophoresis indicating PCR products of TNF-a gene (308G/A) 100 bp marker; 107 bp PCR product and fragments of 87 and 20 bp after digestion

Additionally, CRP may be used to track therapy if the patient has previously been identified as having an infection. CRP levels change depending on the extent of inflammation, and numerous illnesses might result in elevated LDH (Table 1).

The association between TNF-α serum levels and LDH, ESR, and CRP was shown to be non-existent. The TNF-α level was only weakly positively linked with patient age (r=-0.61, p=0.05).

The results of the data analysis comparing the severity, complications, and death caused by COVID-19 in men and women indicated that the incidence of cases with COVID-19 was almost equal between men and women. However, it seems that there is a difference between the two sexes in terms of severity and mortality rate. Evidence showed that the prevalence of severe symptoms (ICU patients) was higher in men than in women.

TNF- is a pro-inflammatory cytokine that can exacerbate disease severity and lung damage in a variety of pulmonary conditions, including COVID-19, asthma, COPD, and ARDS. TNF- can cause cytokine release syndrome (CRS), a potentially fatal systemic inflammatory response that can result in organ failure and death. TNF- α can also facilitate the interaction of SARS-CoV-2 with ACE2 receptors, which are the virus's entry points into lung cells. TNF-inhibitors, which are drugs that block TNF-'s biological actions, may have therapeutic value in slowing disease progression in severe SARS-CoV-2 infection. More clinical trials, however, are required to confirm their safety and efficacy [10,11].

Men were shown to have more severe COVID-19 symptoms than women. The underlying cause of this difference is unclear and could be due to various factors, including behavioral differences such as obesity and addiction, or differences in immunological responses between the sexes [10].

There was no significant difference in the number of genotype alterations between healthy and ill people, although those with the AA genotype had higher blood TNF-α levels.

The possibility that the -308 G > A polymorphism affects the activity of TNF-α gene promoter and that the TNF-α gene shows the same alleles of the genes associated with the major histocompatibility complex (MHC) suggests that immunological response to

the COVID-19 pathogenesis may be affected by this polymorphism [11].

Due to the small sample size, our results may be biased, but previous studies have indicated a relationship between TNF-α -308 G > A gene polymorphism and susceptibility to different kinds of diseases [12].

Covid-19 is still not well-understood as an autoimmune disorder, and genetic factors may play a role in this disease. Therefore, identifying the pathways and genes that render the patient more susceptible to COVID-19 is crucial. As TNF-α affects a wide range of inflammatory and pro-inflammatory processes, changes in its levels may be responsible for the variation in the sensitivity to and severity of COVID-19. Many studies have suggested that TNF- gene promoter polymorphism, which influences mRNA and protein expression levels in various disorders, may affect endogenous TNF- production [13].

Table 1 Demographic and biochemical characteristics COVID-19 patients and healthy control group

Characteristics	Patients with Covid-19	Normal Control
Gender		
- Male	53%	49%
- Female	47%	51%
Age	51.78±4.61	63.24±3.04
ICU	17.07±1.65	----------
History of corona vaccine injection		
- No Shot	19.51±2.96	5.63±3.02
- One dose	2.43±4.01	13.41±2.09
- Two doses	73.17±3.94	78.02±3.12
- Three doses	4.89±2.06	2.94±4.18
History of illness		
- Diabetes		
- Hypothyroidism	24.39±0.98	13.68±1.56
- Respiratory Problems	31.71±1.58	26.34±2.01
- Hypothyroidism	14.63±1.82	7.35±1.05
- Kidney Disease	19.51±1.78	2.09±3.01
- Fatty Liver	12.20±2.15	10.28±2.65
- Mental Problem	9.76±4.01	18.32±3.14
- Cancer	7.32±3.14	3.25±1.09
- No comorbidities disease	2.44±3.21	4.71±1.96
	9.76±2.65	13.98±1.03
Biochemical Tests		
- Erythrocyte sedimentation rate (ESR) (mm/hr)	56.82 ± 8.19	23.34 ± 6.85
- C-Reactive protein (CRP) (mg/l)	46.25 ± 6.09	3.07 ± 4.28
- Lactate Dehydrogenase (LDH) (U/L)	542 ± 32.05	219.02 ± 76.13

Values are presented as mean ± SEM (p<0.05).

According to Saleh *et al.* (2020), patients with COVID-19 who had the AA TNF-α genotype were more likely to experience disease progression and severity [3]. Wang *et al.* (2008) used PCR Sequence-based Typing (PCR-SBT) technology to examine the link between TNF-α gene polymorphisms and SARS infection. They found that the -1031CT/CC and -863 AC genotypes may be associated with femoral head necrosis in SARS patients, but there was no conclusive link between TNF-α gene polymorphisms and SARS infection [7]. Oekelen *et al.* (2021) found that the level of TNF-α in patients' blood at the time of admission is a reliable indicator of the patient's prognosis [6]. Mortaz *et al.* (2021) also reported findings similar to the results of the present study. They investigated TNF-Receptor 1 (sTNFR1) and TNF-α serum levels in mild and severe COVID-19 cases. The measurement of these two factors in the serum of patients can be considered biomarkers for determining the disease severity and the mortality rate of patients [14].

Previous studies have demonstrated the roles of the TNF-α gene and its receptor in the development of COVID-19. These studies have shown that the TNF-α -308 A allele may influence the transcriptional activity that causes increased TNF-α production and more severe responses in COVID-19 patients. Prolonged production of TNF-α caused by TNF-α gene variant may result in severe or uncontrolled inflammation, increasing the risk of respiratory problems [15-18]. Jamil *et al.* (2017) showed that while some of the TNF-α genotypes showed no significant association with diabetes mellitus type II, the TNF-α 308G/A polymorphism was a strong diabetes risk factor in the older age group [8].

Another study found that the TNF-α 1031CC genotype was a risk factor for chronic periodontitis, and the TNF-α 308AA genotype was a risk factor for aggressive periodontitis [19]. Pattanaik *et al.* (2021) reported that Mycobacterium tuberculosis EsxL can induce TNF-α secretion via TLR2 through the activation of the NF-κB and MAPK signaling pathways [20].

Furthermore, TNF-α-induced secretion of intestinal myofibroblast matrix metalloproteinase (MMP) triggers a proteolytic cascade, which may alter intestinal architecture, leading to changes in gut homeostasis and diarrhea [21,22]. Patients with COVID-19 may experience gastrointestinal problems such as diarrhea or vomiting. Previous studies have shown that patients with intestinal disorders benefit from the use of anti-TNF-α monoclonal antibodies. Therefore, it is suggested that patients with COVID-19 who have diarrhea consider using monoclonal antibodies against TNF-α. However, it is challenging to understand the function of internal variables (genetic) and the impact of environmental factors as external components in disorders like COVID-19, which is not yet completely understood. To effectively identify the risk variables affecting such an illness, the use of only one genetic test is insufficient. Interestingly, bioinformatics analysis identified the 308 locations as the binding site for the TFII-I transcription factor family, which has been associated with various neurological, immune, and cancer diseases in previous research [23].

TFII-I downstream of Pol II peaks is involved in the regulation of the globin gene, including activating transcription factor 3 (ATF3) and heme-regulated inhibitor (HRI). TFII-I also affects erythroid-specific gene promoters or cell cycle modulators such as β-globin and

GATA1 [24]. Heme-iron dysregulation is a critical issue observed in individuals with COVID-19 [25, 26], which may be a result of dysregulation of TFII-I transcription factor family in the 308 positions.

In the present bioinformatics analysis, increased COVID-19 susceptibility and the TNF-α -308 G > A polymorphism were associated with each other for the first time. Thus, COVID-19 severity may be associated with the TNF-α -308A genotype. Future studies should examine these findings and assess the connection between COVID-19 development and outcome and the TNF-α -308 G > A polymorphism in the clinical setting.

The detection of a specific set of cytokine gene allele variants, including the TNF- gene in the promoter, as well as measurements of inducible and spontaneous LDH, CRP, and ESR production by cells, may allow us to predict the outcome of COVID-19 patients and assess the efficacy of cytokine therapy.

In conclusion, serum levels of TNF-α were higher in COVID-19 patients than in healthy control subjects, and this was associated with more severe complications. However, no relationship was found between COVID-19 occurrence and these TNF-α gene polymorphisms. Therefore, COVID-19 development may be influenced by other factors. Nonetheless, susceptibility to infection with COVID-19 may be predicted by finding the role these promoter polymorphisms play through experiments on a larger statistical population assessing the transcriptional activity of TNF-α in patients with a specific allele(s) in TNF-α -308. This study can help in deciding therapeutic methods for better treatment of COVID-19. However, demonstrating the clinical utility of the TNF-α -308 G > A polymorphism in COVID-19 diagnosis and treatment requires larger-scale clinical trials.

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Ethics approval and consent to participate

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Conflict of interest

The authors declare that there is no conflict of interest in this study.

Authors' contributions

All authors read and approved the final version of the manuscript, **NA** supervised the project, and edited and co-wrote the manuscript. **SS**; data analysis and wrote the manuscript, **FN and EKR** carried out the part of the research, and **SKF** designed the study performed data analysis and wrote the manuscript.

ORCID

Nahid Askari
https://orcid.org/0000-0002-9462-1144
Sara Shafieipour
https://orcid.org/0000-0002-9341-694X
Fatemeh Noormand
https://orcid.org/0000-0002-4441-6310
Elahe Karami Raviz
https://orcid.org/0000-0003-3289-8823
Soudeh Khanamani Falahatipour
https://orcid.org/0000-0002-3539-8982

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