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Association of tumor necrosis factor alpha -308 single nucleotide polymorphism with SARS CoV-2 infection in an Iraqi Kurdish population

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Abstract

Uncovering risk factors playing roles in the severity of Coronavirus disease 2019 (Covid-19) are important for understanding pathoimmunology of the disease caused by severe acute respiratory syndrome Coronavirus 2 (SARS CoV-2). Genetic variations in innate immune genes have been found to be associated with Covid-19 infections. A single-nucleotide polymorphism (SNP) in a promoter region of tumor necrosis factor alpha (TNF-α) gene, TNF-α −308G>A, increases expression of TNF-α protein against infectious diseases leading to immune dysregulations and organ damage. This study aims to discover associations between TNF-α −308G>A SNP and Covid-19 infection. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for genotyping a general Kurdish population and Covid-19 patients. The homozygous mutant (AA) genotype was found to be rare in the current studied population. Interestingly, the heterozygous (GA) genotype was significantly (p value = 0.0342) higher in the Covid-19 patients than the general population. This suggests that TNF-α −308G>A SNP might be associated with Covid-19 infections. Further studies with larger sample sizes focusing on different ethnic populations are recommended.

KEYWORDS
Covid-19, Iraq, polymorphism, SARS CoV-2, SNP, TNF- 308

1 | INTRODUCTION

Coronavirus disease 2019 (Covid-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), has been considered as a pandemic and public health threat. The immunopathogenesis of Covid-19 requires outstanding research to uncover the reasons behind the severity of the disease in some Covid-19 patients while it is mild or asymptomatic in others. It is known that SARS CoV-2 enters into human cells via interactions between human angiotensin-converting enzyme 2 receptor (ACE2) and viral spike protein.

Additionally, SARS CoV-2 initiates both innate and adaptive immune response through pattern recognition receptors and cytokines, particularly proinflammatory cytokines including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-α), which lead to cytokine storm, acute respiratory distress syndrome (ARDS) and death in some Covid-19 patients.[1,2]

Researchers have been continuously looking for discovering risk factors that can be connected to the disease. At the first step, it was suggested that individuals, who are old, male and have previous cardiovascular problems, severely acquire the disease. Furthermore, it

was also noticed that genetic variations in human genes (e.g., ACE2, TMPRSS2, cytokines, TLR-7, androgen receptor, and protease genes) may also be related to Covid-19 severity.[3] Consequently, candidate genes, including genetic variants in cytokines, chemokines, and receptors, were selected based on their previous associations with SARS Cov-1, SARS CoV-2, and other infectious diseases.[4,5] The candidate genes include variations in ABO blood groups, human leukocyte antigen (HLA loci; type I interferon-related genes (e.g., TLR3, TLR4, UNC93B1, TBK1, IRF7, IFNAR2, OAS, IFITM3, and PRKRA; cytokines (e.g., TNF-α, TMEM189-UBE2V1, IL-17A, and WSB1; chemokines (e.g., CCR2, CCR5, CCR9, CXCR6, and XCR1; and other genes (e.g., DPP7, DPP9, MST1R, GOLGA3, GOLGA8B, LAPTM4b, and ApoE.[2] Consequently, Covid-19 Host Genetics Initiative (HGI) (https://www.covid19hg.org/) announced a global project collaborating among researchers to discover genetic loci in various populations. Analyzing three meta-analysis studies using approximately 50 thousand people from 19 countries, the HGI project has found 13 genetic loci associated with Covid-19.[6] These loci and their closest genes are highlighted (rs2271616: SLC6A20), (rs10490770: LZTFL1), (s11919389: RPL24), (rs1886814: FOXP4), (rs72711165: TMEM65), (rs912805253: ABO), (rs10774671: OAS1), (rs77534576: TAC4), (rs1819040: KANSL1), (rs2109069: DPP9), (rs74956615: RAVER1), (rs4801778: PLEKHA4), and (rs13050728: IFNAR2).[6] Whole-genome sequences of thousand Covid patients have discovered associations between Covid-19 infections and variants in genes including IL10RB and PLSCR (interferon signaling), BCL11A (leukocyte differentiation), FUT2 (blood type antigen secretor status).[7] In a recent systematic review, the TNF-α gene was considered as one of the most important candidate genes which are potentially associated with progressions of Covid-19.[8]

Tumor necrosis factor alpha (TNF-α) gene is located on chromosome 6p21.33 and encodes TNF-a protein secreted by macrophages and expressed in most organ tissue including lungs (https://www.ncbi.nlm.nih.gov/gene/7124). TNF-α SNPs are associated with infectious, autoimmune, dermatological, cardiovascular, neurological diseases.[9] TNF-α −308 G > A (rs1800629) in the promoter region of the TNF gene impacted on TNF gene expression, has been regarded as an important SNP, in which nucleotide guanine 308 (G) is substituted with Adenine (A).[10] This SNP is found to be associated with septic shock in most ethnic backgrounds,[11] and Caucasian populations.[12] It is also linked with infectious diseases such as malaria in Kenya, parasitic infections in Indonesia, Cytomegalovirus in Finland,[13] a positive single-strand RNA Chikungunya virus in Nicaragua,[14] Deng virus in various ethnicities,[15] and hepatitis C virus in India.[16] Genetic studies of cytokines have not only important for understanding the role of SNPs in the pathophysiology of diseases but are also critical for designing immunomodulatory therapies against infectious diseases as Covid-19.[17]

In the Middle East, particularly in Iraq, studying genetic polymorphisms linking with Covid-19 infection has been unobserved. Before the Covid-19 pandemic emerged, our group has been working on genetic polymorphisms in both Toll-like receptor-4 (TLR4) and Apolipoprotein Epsilon (ApoE) in a general Kurdish population[18,30]; Lately, we have found that ApoE4 allele is associated with Covid-19 infection in this population (19 Recent studies have found that TNF-α −308 G > A[20] and TNF receptors[21] are associated with Covid-19 infections in Egyptian and Mexican populations, respectively. Up to our best knowledge, no studies were conducted on the association of this TNF SNP with Covid-19 in other ethnic backgrounds. This study aims to reveal the association of TNF-α −308 G > A SNP with Covid-19 patients in the Iraqi Kurdish population.

2 | MATERIALS AND METHODS

2.1 | Sample collections

One hundred and twenty-five (125) blood samples were also collected in Covid-19 patients, whose real-time reverse transcriptase polymerase chain reaction (rRT PCR) was positive and seeking treatment in Kalar Polyclinics, Kurdistan Regional Government, Iraq. The unvaccinated, symptomatic, and Iraqi Kurdish patients

TABLE 1 Clinical and demographic characteristics of Covid-19 patients

Parameters		Comorbidities		Demographic data	
Parameter	No. (%)	Comorbidity	No. (%)	Age	
High ESR	89 (71.2)	Obesity	23 (18.4)	Age groups	No. (%)
Positive CRP	103 (82.4)			Below 45 years	55 (44.0)
High D-Dimer	39 (31.2)	Asthma	4 (3.2)	Above 45 years	70 (56.0)
High Ferritin	43 (34.4)			Total	125 (100)
CT scan	31 (24.8)	Diabetes	11 (8.8)	Sex	
Moderate	17 (13.6)			Gender	No. (%)
Severe	14 (11.2)	Hypertension	36 (28.8)	Male	58 (46.4)
SpO$_2$		Ischemic heart disease	10 (8.0)	Female	67 (53.6)
Higher than 93	66 (52.8)				
Lower than 93	59 (47.2)	Stroke	1 (0.8)	Total	125 (100)

were included in this study. Both verbal and written consent forms were taken from the patients in a questionnaire form. The patients' clinical information is shown in Table 1. In the retro-perspective studies performed by our group[18,30]; one hundred and fourteen (114) EDTA conserved blood samples (3 ml) were taken in a general Iraqi Kurdish population as a reference control. This study was ethically approved by the Department of Biology, University of Garmian, Kurdistan Region. The ethical approval committee adheres to the ethical principles of the Declaration of Helsinki for human subjects.

2.2 | Genomic nucleic acid extraction

Total genomic DNA was extracted from the blood samples as described by the manufacturer's instructions (GENET BIO), 200 μl of DNA was acquired for each sample and kept at −20°C) until polymerase chain reaction and genotyping were performed.

2.3 | Polymerase chain reaction (PCR)

A partial DNA sequence of the TNF-α gene, where −308G>A SNP is located, was amplified using a pair of primers as previously described[22] and shown in Table 2. The PCR reaction was as follows: PCR master mix (10 μl) (AddBIO), genomic DNA (4 μl), 10 μM of each primer (0.5 μl). The PCR condition was as follows: initial denaturation at 95°C for 10 min; 35 cycles of (95°C for 45 s, 65°C for 60 s and 72°C for 60 s); and final extension step at 72°C for 10 min using a thermal cycler (Mastercycler nexus, Eppendorf AG). The amplified PCR product (195 bp) was checked on 3% agarose gel and kept at −20°C until genotyping was conducted.

2.4 | Genotyping of TNF-α −308G>A

The amplified PCR products were digested using NcoI restriction enzyme (NEB). The reactions were performed as follows: molecular grade water (10 μl), NcoI buffer (4 μl), NcoI enzyme (10 units) and PCR product (10 μl) incubated at 37°C for 3 h using the thermal cycler. The digested PCR products were inspected on 3% agarose gel. The mutant genotypes were repeated at least twice.

2.5 | Statistical analysis

The differences in the frequency of the TNF-α genotypes and alleles between both Covid-19 and general population samples were analyzed using Chi-square (Fisher's exact test) and t test (Graphpad prism 9.1.1 software GraphPad Software). p-values of more than 0.05 were regarded as statistically significant and both odds ratio and confidence interval (% 95 CI) were also used.

3 | RESULTS

The Covid-19 patients were mostly symptomatic which were indicated by clinical parameters including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), D-dimer, Ferritin, CT scan, and SpO2 (Table 1). More than half of the patients had comorbidities including obesity, asthma, diabetes, hypertension, ischemic heart disease, and stroke. Among the comorbidities, both hypertension and obesity were the most common in the Covid-19 patients. Data also contained both old and young ages, males and females.

The PCR products of both the general population and Covid-19 patients were observed on agarose gel electrophoresis. As shown in Table 2, a single PCR product band, 195, 173, and 195 bp appeared for each the undigested, homozygous wildtype (GG), and homozygous mutant (AA) genotypes, respectively. Also two bands, 173 and 195 pb appeared for the heterozygous (GA) genotype. As shown in Table 3, the heterozygous genotype (GA) of TNF-α −308G>A was significantly higher in Covid-19 patients than the general population, whereas other genotypes and alleles were not statistically significant. There are no significant differences between the ratio of males to females between both the general population and COVID-19 patients. However, there is a highly significant difference between the ages of the studied groups.

Comparisons of both genotypes and allele frequencies between mild and moderate-severe cases were also non-significant (Table 4). Similarly, no statistically significant differences were found in both genotypes and allele frequencies between genders (Table 5). Based on ages, data were broken down to young ages (<45 years old) and old ages (>45 years old) and the results showed that there are no statistical differences between young and old ages (Table 6). As displayed in Table 7, there were no significant differences in both genotypes and allele frequencies in TNF-α −308G>A in Covid-19 patients who had comorbidities or with no comorbidities.

TABLE 2 Primers used for genotyping TNF-α −308G>A PCR products digested by NcoI restriction enzyme

Primers	Sequences 5′–3′	Undigested (bp)	Homozygous Wildtype (bp) GG	Homozygous mutant (bp) AA	Heterozygous (bp) GA
TNF-F	GGAGGCAATAGGTTTTGAGGGCCAT				
TNF-R	CTGTCT-CGGTTTCTTCTCCATGGCG	195	173	195	173 and 195

Note: Sizes of the PCR products (bp) are shown.
Abbreviations: bp, base pair; TNF-F, forward primer; TNF-R, reverse primer.

TABLE 3 Genotypes and allele frequencies of TNF-α −308G>A in the general population and Covid-19 patients

Genotypes	General population n = 114	COVID−19 n = 125	p-Value	Odds ratio	95% CI[a]
Gender	Male (46), female (68)	Male (58), female (67)	0.4365	0.8103	0.4826–1.350
Age	Mean + SD[b] (25.92 + 7.30)	Mean + SD (49.19 + 16.8)	<0.0001	df[c] = 226	19.91–26.64
GG	93	87	0.4314	1.172	0.7939–1.735
GA	17	37	0.0342[*]	0.5038	0.2696–0.9417
AA	4	1	0.2004	4.386	0.7089–54.04
Alleles	General population n = 228	COVID−19 n = 250	p-Value	Odds ratio	95% CI
G	203	211	0.7370	1.055	0.8119–1.371
A	25	39	0.2300	0.7029	0.4189–1.205

[a]Confidence intervals.
[b]Standard deviation.
[c]Degree of freedom.
*Significant.

TABLE 4 Genotypes and allele frequencies of TNF-α −308G>A between mild and moderate-severe Covid-19 patients

Genotypes	Mild N = 73	Moderate-severe n = 52	p-Value	Odds ratio	95% CI
GG n = 87	51	36	>0.9999	1.009	0.5768–1.786
GA n = 37	21	16	0.8523	0.9349	0.4608–1.987
AA n = 1	1	0	>0.9999	2.143	0.08561–53.64
Alleles	Mild n = 146	Moderate-severe n = 104	p-Value	Odds ratio	95% CI
G n = 211	123	88	>0.9999	0.9954	0.6870–1.442
A n = 39	23	16	>0.9999	1.016	0.5159–2.001

TABLE 5 Genotypes and allele frequencies of TNF-α 308G>A between genders

Genotypes	Male N = 58	Female n = 67	p-Value	Odds ratio	95% CI
GG n = 87	42	45	0.8888	1.078	0.6221–1.867
GA n = 37	16	21	0.8513	0.8801	0.4157–1.778
AA n = 1	0	1	>0.9999	0.000	0.000–10.55
Alleles	Male n = 116	Female n = 134	p-Value	Odds ratio	95% CI
G n = 211	100	111	0.8518	1.041	0.7195–1.505
A n = 39	16	23	0.6056	0.8036	0.4182–1.550

TABLE 6 Genotypes and allele frequencies of TNF-α 308G>A between young and old ages

Genotypes	Age below 45 N = 55	Age above 45 n = 70	p-Value	Odds ratio	95% CI
GG n = 87	40	47	0.7809	1.083	0.6217–1.881
GA n = 37	15	22	0.8504	0.8678	0.3993–1.764
AA n = 1	0	1	>0.9999	0.000	0.000–11.62
Alleles	Age below 45 n = 110	Age above 45 n = 140	p-Value	Odds ratio	95% CI
G n = 211	95	116	0.8511	1.042	0.7182–1.512
A n = 39	15	24	0.6033	0.7955	0.4031–1.541

TABLE 7 Genotypes and allele frequencies of TNF-α 308G>A in Covid-19 patients according to comorbidities

Genotypes	No comorbidities N = 67	With comorbidities 45 n = 58	p-Value	Odds ratio	95% CI
GG n = 87	46	41	>0.9999	0.9712	0.5611–1.686
GA n = 37	20	17	>0.9999	1.018	0.5032–2.117
AA n = 1	1	0	>0.9999	Infinity	0.09477 to Infinity
Alleles	No comorbidities n = 134	With comorbidities n = 116	p-Value	Odds ratio	95% CI
G n = 211	112	99	0.9256	0.9793	0.6772–1.417
A n = 39	22	17	0.8631	1.120	0.5811–2.275

4 | DISCUSSION

This study aimed to find associations of TNF-α −308G>A SNP with Covid-19 infection. The results of this study showed that the heterozygous (GA) genotype (29.6%) was significantly (p-value = 0.0342) associated with Covid-19 infection (Table 3). Saleh, et al.[20] have reported that the mutant homozygous (AA) genotype (80.0%), but not the wildtype homozygous (GG) genotype, is predominantly higher in severe Covid-19 patients compared with the heterozygous (GA) genotype (41.7%). In our study, only one homozygous mutant (AA) genotype (0.8%) was unexpectedly found in a mild case who was female, old without comorbidities. It appears that the AA genotype is more prevalent in the Egyptian population studied by Saleh et al.[20] than the Kurdish population in the current study. The homozygous mutant (AA) genotype tends to be rare in the Kurdish ethnic background. Therefore, other ethnic populations should be investigated for TNF-α −308G>A SNP in association with Covid-19. In a Mexican population, TNF receptors (TNFR1 and TNFR2) are associated with Covid-19 and, both soluble TNFR1 and TNFRSF1A:rs767455 variants are high in severe patients.[21] In the current study, the frequency of TNF-α genotypes and alleles, comparing between mild and moderate/severe groups were not statistically significant (Table 4). Similarly, no significant differences were found according to genders (Table 5), ages (Table 6), comorbidities (Table 7). Our cohort data are adequate to support that the heterozygous (GA) genotype, which was statistically significant in comparing between patient and control groups, might be associated with Covid-19 susceptibility (Table 3).

Studies showed that pro-inflammatory cytokines circulating in plasma, particularly TNF-α, are associated with severe Covid-19 infection,[23–26] while others do not support this.[27,28] However, genetic polymorphisms have not been taken into consideration in these studies. Thus, genetic studies along with both serological tests and in vitro studies should be combined in the future to solve these discrepancies. Gene knockdown mouse studies showed that TNF-α acts as a pro-inflammatory cytokine in an initial response to infections, however, it plays also an immunoregulatory role post-infection.[13] It has been observed that most severe or symptomatic Covid-19 patients usually have an initial pro-inflammatory response which is then followed by immune dysregulations

reducing hyperinflammation, cytokine storm, and ARDS in severe patients. Genetic defects in genes (e.g., TNF-α −308 SNP) may modify inflammatory profiles that lead to severe outcomes in severe Covid-19 patients. Thus, studying polymorphisms in the promoter region of the TNF-α gene that enhances its expression will broaden our knowledge on why some Covid-19 patients are more susceptible to SARS CoV-2. It could be hypothesized that the TNF SNP plays a role in the increasing secretion of a high amount of TNF-α protein in severe Covid-19 patients that cause immune dysregulation and organ damage.

The limitations of the current study were that we have not tested other SNPs in the promoter region of the TNF-α gene. It is intriguing to report the co-occurrence of other TNF-α SNPs in severe Covid-19 cases who are young and have no other diseases. It is recommended that young healthy individuals suffering from severe Covid-19 should be included in studies for discovering rare genetic variants which play roles in the severity. A recent study has reported TLR7 genetic variants in severe young male Covid-19 patients with no comorbidities.[29] It seems more interesting to perform whole genome or exome sequencing in young case reports who were previously healthy and currently suffered from severe Covid-19 for exploring multi genetic variations in the same patient.

5 | CONCLUSION

In the current study, the heterozygous genotype of the TNF-α −308 G>A SNP was significantly higher in the Covid-19 patient group in an Iraqi Kurdish population compared with that of the general population. This suggested that this SNP might be associated with Covid-19 infection. However, a larger number of samples are needed to confirm this. Investigations of other TNF-α SNPs are recommended for future studies including 1031T>C, 863C>A, 857C>A, 851C>T, 419G>C, 376G>A, 238G>A, 162G>A, and 49G>A.

CONFLICT OF INTEREST

All authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

HNA, SSN, and SMAA carried out conceptualization, design analysis, planning, and editing the manuscript. HNA carried out sample collections and clinical data. SSN and SMAA carried out genotyping and drafting the article. SMAA carried out statistical analysis.

DATA AVAILABILITY STATEMENT

All data are available through corresponding author, Sherko S. Niranji.

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