Cytotoxic T-lymphocyte-associated antigen 4 *(CTLA-4)* +49A>G (rs231775) gene polymorphism is not associated with COVID-19 severity and mortality in an Iranian population

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ABSTRACT

Introduction: Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates T cell immune responses as an immune activation inhibitor. Literature reviews suggest that COVID-19 is associated with dysregulation of the inflammatory immune response. The purpose of the present hospital-based case-control study was to evaluate the genetic association of the CTLA4 +49A > G (rs231775) Single Nucleotide Polymorphism (SNP) with COVID-19 severity and mortality among the Iranian people.
Method: Genomic DNA of peripheral blood nuclear cells was extracted from the 794 COVID-19 patients and 167 control individuals. The polymorphic site of rs231775 was genotyped using the PCR-RFLP technique. Also, to identify whether this genetic variation was related to *CTLA-4* mRNA expression, total RNA was extracted from 178 COVID-19 patients and 70 controls. The mRNA levels of *CTLA-4* were determined using real-time PCR.
Result: There were no statistically significant differences found in the genotype and allele frequencies among the different genetic models with regards to the severity and mortality of COVID-19. Furthermore, there was no significant association between rs231775 genotypes and CTLA-4 mRNA expression in patients.
Conclusion: Our findings demonstrated that SARS-CoV-2 infection is not associated with rs231775 in the Iranian people. More investigations are crucial to show how this genetic variation affects other ethnic groups. Given the importance of CTLA-4 in regulating immune responses, further studies are recommended to examine other CTLA-4 SNPs and the function of this gene in COVID-19 patients.

1. Introduction

As of late December 2019, coronavirus disease 2019 (COVID-19) was classified as a threat to the world's public health [1]. As of

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March 2023, over 761 million people had been infected, and over 6.8 million individuals had died from the disease globally [2]. The severity of clinical symptoms of COVID-19 ranges from asymptomatic to progressive, life-threatening, and organ failure [3]. Despite the extensive studies conducted in this field, the pathogenesis of COVID-19 is still not completely understood. Several lines of evidence indicate that the quality and quantity of immune responses, especially T lymphocytes, are related to the replication rate of the virus and disease severity [4]. Also, it has been reported the host's overactive immune response, which is unable to eradicate the virus, intensifies respiratory distress and cardiac dysfunction and harms other organs [5,6].

The Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, also called CD152), an inhibitory immune checkpoint molecule, is located on chromosome 2q33. It contains four exons and encodes 233 amino acids [7,8]. During T cell signaling, CTLA-4 is expressed on activated T cells and down-regulates T cell immune responses by inhibiting T-cell activation, cytokines production, and inducing Fas-independent apoptosis of activated T cells. So, CTLA-4 maintains T-cell homeostasis, and impaired CTLA-4 expression may lead to an imbalance in immune homeostasis [9,10].

Host genetics has the potential to influence one's susceptibility to a disease, as well as its subsequent ability to mount an effective immune response [11]. The COVID-19 Host Genetics Initiative (HGI) meta-analysis reported 23 genomic loci significantly related to COVID-19 susceptibility or severity by investigating 125,584 COVID-19 patients and 2,500,000 healthy controls from 60 Genome-Wide Association Studies (GWAS) in 25 countries [12]. Because the prevalence and mortality rate of COVID-19 varies widely between countries, it has been hypothesized that interactions between environmental factors and host genetic variation may influence disease outcomes [13,14]. Previous studies on COVID-19 Iranian patients demonstrated that the COVID-19 mortality rate is correlated with some single nucleotide polymorphisms (SNPs) as a host genetic variation in Interferon Induced Transmembrane Protein 3 (IFITM3), angiotensin-converting enzyme 2 (ACE2), interferon lambda 3/4 (IFNL3/4), transmembrane serine protease 2 (TMPRSS2), ABO blood group antigens, and Interleukin 10 (IL10) [15–21]. Some investigations showed single-nucleotide polymorphisms in the *CTLA-4* gene may affect T-cell mediated anti-viral immune responses [22,23]. +49A > G (rs231775) is a common polymorphism in the *CTLA-4* gene region. This SNP, as a non-synonymous polymorphism, is located at the first exon of *CTLA-4*, leading to 17Thr-to-Ala amino acid substitution in the sequences of peptide leader [24]. Thr/Ala amino acid substitution leads to incomplete glycosylation in the endoplasmic reticulum. Because of this, the amount of CTLA-4 (Ala 17) on the cell surface is reduced [25]. Previous studies suggested this common polymorphism might influence the binding of CTLA-4 to its ligands, B-7 molecules, leads reduce inhibitory function and expression of CTLA-4 on activated T cells [7,26]. Thus, rs231775 could affect transcriptional and translational activity through alternative splicing and 17Thr>17 Ala substitution, respectively. CTLA-4 rs231775 has been described to be associated with a higher risk of several immune-related disorders, including Asthma, chronic bronchitis, autoimmune diseases (such as type 1 diabetes, Graves' disease, Behçet's disease, Hashimoto's thyroiditis, rheumatoid arthritis, systemic lupus erythematosus), Hepatitis B Virus, and Hepatitis C Virus between different studied populations [26–35]. Therefore, selection criteria for CTLA-4 rs231775 polymorphism included its functional properties in different clinical conditions and minor allele frequency (MAF) greater than 20 % for the South Asian population.

Considering the significant role of CTLA-4 in regulating inflammatory immune responses, in the present study, as far as we know, for the first time we aimed to determine the association of CTLA-4 +49A/G (rs231775) polymorphism with the development, severity, and mortality of COVID-19 in the Iranian people. We also examined mRNA levels of *CTLA-4* in the patients carrying different genotypes of the CTLA-4 rs231775 polymorphism.

2. Materials and methods

2.1. Study population

This study was conducted on 794 COVID-19 patients and 167 subjects as controls between January and June 2020 in Iran. The inclusion criteria included confirmed COVID-19 infection by real-time polymerase chain reaction (real-time PCR) test, chest computed tomography imaging (only for inpatients), and clinical assessment by an expert specialist (infectious disease). Also, exclusion criteria involved missing specimens and non-Iranian patients. According to the sampling performed during the first peak of SARS-CoV-2 infection in Iran, none of the studied patients had a previous background of COVID-19 infection or vaccination against this disease. The 794 definite COVID-19 cases (determined by the WHO Interim Guidance and Comprehensive National Guideline for the Diagnosis and Treatment of COVID-19, 6th edition) [36] were categorized into four groups based on the severity of their condition: mild (n = 295, out-patients with infection-related symptoms), moderate (n = 384, hospitalized patients with infection-related symptoms), severe (n = 37, intensive care unit (ICU) admitted patients with at least one of the following conditions: Oxygen saturation ≤93 % at rest, respiratory rate≥30 times/min, and PaO2/FiO2 ≤ 300 mmHg), and critical (n = 78, ICU-admitted patients with at least one of the following conditions: intubation due to respiratory failure, multiple organ failure, and shock) subgroups. As a control group, one hundred sixty-seven healthy subjects (without previous background of COVID-19 infection and underlying diseases) from the same state with comparable ethnic circumstances were recruited. Informed consent was acquired. The consent was obtained from the companions of patients suffering from severe or critical illness. The National Ethics Committee on Medical Sciences Research of the Iranian Ministry of Health and Medical Education approved this study (IR.SHAHED.REC.1400.046).

2.2. DNA extraction and rs231775 genotyping

According to the manufacturer's instructions, genomic DNA was extracted using the GeneAll® ExgeneTM kit (South Korea) from peripheral venous blood (3–5 ml) collected from each participant in an EDTA-pretreated tube; then, extracted DNA was stored at

−20 °C.

The CTLA-4 +49A/G polymorphism (rs231775) in the first exon region of *CTLA-4* was genotyped by the PCR-RFLP method. Briefly, forward (5′-ACCCACGGCTTCCTTTCTC-3′) and reverse (5′-ACACCTCCTCCATCTTCATGC-3′) primers were used to amplify the DNA sequence of interest to generate the 358 bp *CTLA-4* amplified DNA segment. The mixture of reaction (20 μl) made up of TEMPase master mix (10 μl) (AMPLIQON), primers (10 pM), and genomic DNA (0.02–0.04 μg). PCR thermocycler (Bio-Rad, USA) was used to amplify the genomic DNA, following a program including initial denaturation (95 °C for 15 min), followed by 35 denaturation cycles (95 °C for 30 s), annealing (55 °C for 30 s), extension (72 °C for 30 s) and final extension (72 °C for 5 min).

To identify the CTLA-4 +49A/G, the PCR products (358 bp) were subjected to digestion with allele-specific restriction endonuclease BseXI (Bbv-I, ThermoScientific, USA). Briefly, a reaction mixture including *Bse*XI enzyme (1 μL), related buffer (2 μL), nuclease-free water (18 μL), and PCR products (10 μL) was incubated for 16 (hrs.) at 65 °C; Then, the reaction was inactivated at 80 °C for 20 min. The DNA-digested fragments were separated using gel electrophoresis on a 2 % agarose gel. In a gel documentation system, ultraviolet illumination was used to visualize the DNA fragments. The restriction site of DNA digestion is present in G allele. The G allele was digested into fragments of 243 and 114 bp, while the A allele remains intact (Supplementary fig. 1).

Sanger sequencing was employed to validate the precision of genotyping outcomes for a subset comprising 10 % of the samples (Supplementary fig. 2). The genotyping results were all in agreement.

Table 1
Demographic characteristics of COVID-19 patients (categorized by disease severity).

	COVID-19 patient (n = 794)			Healthy control (n = 167)
Age (year)	52 ± 17Φ			45 ± 13
Gender				
Female	305 (38.4)			63 (37.7)
Male	489 (61.6)			104 (62.3)
COVID-19 patients				
	Mild (n=295) **Mean±SD**	**Moderate (n=384)** **Mean±SD**	**Severe (n=37)** **Mean±SD**	**Critical (n=78)** **Mean±SD**
Age (year)	45 ± 14***,###,$$$	56 ± 16 ††	58 ± 17	62 ± 15
T (°C)	36.94 ± 0.34 **,$$	37.19 ± 0.76	37.02 ± 0.62	37.28 ± 0.85
PR (beats/minutes)	91.19 ± 11.44 $	90.48 ± 14.45 †††	90.41 ± 13.2 ‡	97.81 ± 16.93
RR (beats/minutes)	24 ± 5	23 ± 6 ††	24 ± 8	25 ± 6
SBP (mm Hg)	125.3 ± 22.73	121.84 ± 17.84 †	122.46 ± 25.94	128.21 ± 23.43
DBP (mm Hg)	76.7 ± 9.09	75.44 ± 11.53	74.92 ± 13.57	76.79 ± 13.88
SpO2 (%)	91 ± 8 ##,$$	90 ± 7 ψψ,†††	86 ± 8	84 ± 11
Gender				
Female	118 (40)	148 (38.6)	12 (32.4)	27 (34.6)
Male	177 (60)	236 (61.4)	25 (67.6)	51 (65.4)
Comorbidities				
Hypertension				
Yes	37 (14.7)	118 (33.8)	19 (45.2)	27 (34.6)
Diabetes mellitus				
Yes	29 (11.6)	95 (27.2)	18 (42.9)	29 (37.2)
cancer				
Yes	2 9 (0.8)	11 (3.2)	0	6 (7.7)
Heart Disease				
Yes	12 (4.8)	67 (19.2)	11 (26.2)	18 (23.1)
Respirotry Disease				
Yes	11 (4.4)	29 (8.3)	5 (11.9)	12 (15.4)
Renal Disease				
Yes	11 (4.4)	26 (7.4)	5 (11.9)	12 (15.4)
liver Disease				
Yes	3 (1.2)	7 (2)	2 (4.8)	1 (1.3)
thyroid Disease				
Yes	14 (5.6)	10 (2.9)	1 (2.4)	7 (9)
Cerebrovascular accident				
Yes	0 (0)	9 (2.6)	1 (2.4)	5 (6.4)

Data are presented as Mean ± SD or n (%) as appropriate.
Φ $P < 0.05$, P-value stand for the comparison between COVID-19 patients and Healthy controls.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, P-value stands for the comparison between Mild patients and Moderate patients. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, P-value stands for the comparison between Mild patients and Severe patients.
\$ $P < 0.05$, \$\$$P < 0.01$, \$\$\$$P < 0.001$, P-value stands for the comparison between Mild patients and Critical patients. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$, P-value stands for the comparison between Moderate patients and Critical patients.
ψ $P < 0.05$, ψψ $P < 0.01$, ψψψ $P < 0.001$, P-value stands for the comparison between Moderate patients and Severe patients.
‡$P < 0.05$, ‡‡$P < 0.01$, ‡‡‡$P < 0.001$, P-value stands for the comparison between Severe patients and Critical patients.
Abbreviations: T (Temperature), PR (pulse rate), RR (Respiratory rate), SBP (systolic blood pressure), DBP (diastolic blood pressure), SpO2 (Peripheral oxygen saturation).

2.3. RNA extraction and CTLA-4 expression

A Real-time PCR technique was performed to analyze the relationship between the rs231775 polymorphism and *CTLA*-4 mRNA expression in 178 COVID-19 patients (Mild = 62, Moderate = 64, Severe = 15, Critical = 37) and 70 healthy participants without underlying disease. Because of the small number of patients in the higher severity subgroups, a stratified sampling method has been used to select the sample for mRNA level evaluation. Total RNA was extracted from 3 ml of peripheral venous blood collected in EDTA-pretreated tube using GeneAll® Hybrid-R ™ kit (South Korea) according to the manufacturer's guideline. The purity and concentration of the total extracted RNA were quantified by spectrophotometer (ThermoScientific, USA). Then, the integrity of extracted RNA was assessed using a bioanalyzer (ABI, USA). Using a cDNA synthesis kit (ABI, USA), 1000 ng of extracted RNA was converted into complementary DNA (cDNA) and stored at −20 °C. The final volume of the reaction mixture was 20 μl containing 10 μl of SYBER Green Real-Time PCR master mix (AMPLIQON, Denmark), 2 μl 1:10 diluted cDNA, 5 pM of primers (forward primer, 5′- GACCTGAA-CACCGGCTCCCAT -3′, and reverse primer, 5′- ACACACAAAGCTGGCGATGC -3′), and 6 μL Nuclease-free distilled water. Notably, each sample was examined in triplicate. The StepOne™ Real-Time PCR System (Applied Biosystems, USA) was used to perform the PCR as follows: an initial 95 °C denaturation for 16 min followed by 40 cycles of denaturation at 95 °C for 30 Seconds, annealing at 61 °C for 60 Seconds and extension at 72 °C for 30 Seconds. Expression of human β-actin was measured as an internal reference gene. The sequences of β-actin primers were 5′- CATCGAGCACGGCATCGTCA -3′ (forward) and 5′-TAGCACAGCCTGGATAGCAAC -3′ (reverse). Finally, the relative mRNA expression ratio of *CTLA-4* to β-actin between study groups was determined using the Pfaffl method [37].

2.4. Statistical analysis

The allelic and genotypic frequencies of CTLA-4 rs231775 were identified by Pearson's chi-square test on SPSS software, version 26.0 (USA); and also online SNPstats software. As well, the relationship between the rs231775 genotypes and COVID-19 severity and mortality was demonstrated using OR (odds ratios) and 95 % CI (confidence intervals) in various genetic models. We also examined deviations from the Hardy-Weinberg balance between study groups using the online SNPstats software. *CTLA*-4 mRNA expression stratified by the rs231775 polymorphic genotypes was analyzed by the Mann–Whitney *U* test, and a P-value ≤0.05 was selected to determine statistical significance. GraphPad Prism 8, version 8.0.1 was used to present all graphs.

3. Results

3.1. Demographic characteristics

The demographic characteristics of the study groups are shown in Table 1. 794 COVID-19 cases (489 men and 305 women) with different severity of disease (mild (n = 295), moderate (n = 384), severe (n = 37), and critical (n = 78)) were enrolled in this study. Compared to the controls (n = 167), the mean age of the patients was significantly higher than controls (P ≤ 0.001). The age of the patients increased steadily but considerably from the mild to critical cases. According to medical records on admission, critical patients' respiratory rates were significantly higher than those of moderate patients (P = 0.003). Furthermore, the oxygen saturation in severe and critical patients was significantly lower than in the mild (p = 0.004 and P = 0.002) and the moderate (P = 0.003 and P ≤ 0.001) cases. The temperature was significantly higher in the critical and moderate cases than in the mild ones (P = 0.002 and P = 0.007). In addition, the heart rate was significantly higher in the critical cases than the mild, moderate, and severe ones (P = 0.03, P ≤ 0.001, and P = 0.019, respectively). The systolic blood pressure was significantly higher in critical patients than the moderate ones (P

Table 2
The frequency of the rs231775 genotypes among COVID-19 patients with different severity under hereditary models' analysis.

Genotypes	COVID-19 patients	Healthy control	OR (95 % CI)	P-Value	Mild	Moderate	Severe	Critical
Codominant model, n (%)								
AA	392 (49.4)	89 (53.3)	1	0.65	151 (51.2)	190 (49.5)	16 (43.2)	35 (44.9)
AG	329 (41.4)	64 (38.3)	1.17(0.82–1.66)		115 (39)	166 (43.2)	16 (43.2)	32 (41)
GG	73 (9.2)	14 (8.4)	1.18 (0.64–2.19)		29 (9.8)	28 (7.3)	5 (13.5)	11 (14.1)
Dominant model, n (%)								
AA	392 (49.4)	89 (83.3)	1	0.36	151 (51.2)	190 (49.5)	16 (43.2)	35 (44.9)
AG + GG	402 (50.6)	78 (46.7)	1.17 (0.84–1.63)		144 (48.8)	194 (50.5)	21 (56.8)	43 (55.1)
Recessive model, n (%)								
AA + AG	721 (90.8)	153 (91.6)	1	0.74	266 (90.2)	356 (92.7)	32 (86.5)	67 (85.9)
GG	73 (9.2)	14 (8.4)	1.11 (0.61–2.01)		29 (9.8)	28 (7.3)	5 (13.5)	11 (14.1)
Overdominant, n (%)								
AA + GG	465 (58.6)	103 (61.7)	1	0.46	180 (61)	218 (56.8)	21 (56.8)	46 (59)
AG	329 (41.4)	64 (38.3)	1.14 (0.81–1.60)		115 (39)	166 (43.2)	16 (43.2)	32 (41)
Allele frequency								
A	1113 (70.1)	242 (72.5)	1	0.39	417 (70.7)	546 (71.1)	48 (64.9)	102 (65.4)
G	475 (29.9)	92 (27.5)	1.123 (0.863–1.460)		173 (29.3)	222 (28.9)	26 (35.1)	54 (34.6)

Data are presented as n (%) as appropriate.
Abbreviations: ORs, odds ratio.

= 0.034). Diastolic blood pressure did not differ significantly between the study groups. The study groups were well-matched in terms of gender (P = 0.977). Regarding Table s1 and In COVID-19 patients, the most prevalent underlying diseases were hypertension (25.3 %), diabetes mellitus (21.5 %), coronary heart disease (13.6 %), respiratory diseases (7.18 %), renal disorder (6.8 %), cancer (5.8 %), thyroid disorders (4.03 %), cerebrovascular accidents (1.89 %), and liver-related diseases (1.6 %).

3.2. Genotype distributions of CTLA-4 +49A > G (rs231775) in COVID-19 patients

We genotyped rs231775 in patients with COVID-19 to analyze the influence of the +49A > G polymorphism on the susceptibility to infection. The frequencies of this SNP were consistent with the Hardy-Weinberg equilibrium in the control group (P = 0.57). The minor allele frequency (MAF) of rs231775 in the control group was 0.27; this value is similar to the data reported (0.3) in the 1000 Genomes project of the South Asian population (http://www.ncbi.nlm.nih.gov/projects/SNP/). The allele and genotype frequency distributions among the different genetic models did not show significant differences between study groups, as shown in Table 2. Also, the re-lationships between demographic factors like age, gender, clinical manifestation, and underlying disease stratified by rs231775 ge-notypes were examined in COVID-19 patients. There were no significant differences in COVID-19 patients' age, gender, and comorbidities relating to rs231775. Among clinical manifestations, only pharyngitis in patients with the GG genotype was significantly higher than the others (P = 0.018).

As noted above, there was a significant difference in the mean age of the study groups. Further analysis after dividing the age groups into <40, 40–80, and >80 years showed that the age disparity had no confounding influence on the results.

3.3. Association of CTLA-4 +49A > G (rs231775) with the severity of COVID-19

To explore the impact of the +49A > G (rs231775) on the disease progression, we also examined the rs231775 genotypes in COVID-19 cases in the range of disease severity. As shown in Table 2, multivariate logistic regression analysis did not find any significant differences between groups. Also, based on further analysis, there were no significant differences in allele and genotype frequencies of this polymorphism between ICU (Severe + Critical) and non-ICU (Mild + Moderate) admitted patients under different inheritance models (Table 3). Notably, demographic parameters, including age, gender, and underlying disease were stratified by rs231775 ge-notypes. There were no significant differences relating to rs231775.

3.4. Association of CTLA-4 +49A > G (rs231775) with the mortality of COVID-19

Table 4 shows the effect of rs231775 on COVID-19 mortality. There were no significant differences in allele and genotype frequency distributions between survivors and non-survivors under different inheritance models. Notably, demographic parameters like age, underlying diseases, and gender were classified according to genotypes of rs231775. There were no significantly variations relating to rs231775.

3.5. Impact of rs231775 on CTLA-4 mRNA expression in COVID-19 patients

The CTLA-4 mRNA levels in COVID-19 cases were significantly lower than in healthy controls. The relative CTLA-4 mRNA expression in patients with different genotypes of rs231775 were 0.87 ± 0.55 (AA), 0.82 ± 0.49 (AG), and 0.85 ± 0.6 (GG). Further statistical analysis showed none of the rs231775 genotypes were associated with the CTLA-4 mRNA expression (Fig. 1).

Table 3
The frequency of the rs231775 genotypes among non-ICU and ICU-admitted COVID-19 patients under hereditary models' analysis.

Genotypes	non-ICU-admitted patient (Mild + Moderate) (n = 679)	ICU-admitted patient (Severe + Critical) (n = 115)	OR (95 % CI)	P-Value
Codominant model, n (%)				
AA	341 (50.2)	51 (44.4)	1	0.17
AG	281 (41.4)	48 (41.7)	1.14 (0.75–1.75)	
GG	57 (8.4)	16 (13.9)	1.88 (1–3.52)	
Dominant model, n (%)				
AA	341 (50.2)	51 (44.4)	1	0.24
AG + GG	338 (49.8)	64 (55.6)	1.27 (0.85–1.88)	
Recessive model, n (%)				
AA + AG	622 (91.6)	99 (86.1)	1	0.073
GG	57 (8.4)	16 (13.9)	1.76 (0.97–3.19)	
Overdominant, n (%)				
AA + GG	398 (58.6)	67 (58.3)	1	0.94
AG	281 (41.4)	48 (41.7)	1.01 (0.68–1.52)	
Allele frequency				
A	963 (70.9)	150 (65.2)	1	0.081
G	395 (29.1)	80 (34.8)	0.77 (0.57–1.03)	

Data are presented as n (%) as appropriate.
Abbreviations: ORs, odds ratio.

Table 4
The frequency of the rs231775 genotypes among survivor and non-survivor COVID-19 patients under hereditary models' analysis.

Genotypes	Survivor	non-survivor	OR (95 % CI)	P-Value
Codominant model, n (%)				
AA	353 (49.6)	39 (47.6)	1	0.61
AG	296 (41.6)	33 (40.2)	1.01 (0.62–1.64)	
GG	63 (8.8)	10 (12.2)	1.44 (0.68–3.03)	
Dominant model, n (%)				
AA	353 (49.6)	39 (47.6)	1	0.73
AG + GG	359 (50.4)	43 (52.4)	1.08 (0.69–1.71)	
Recessive model, n (%)				
AA + AG	649 (91.2)	72 (87.8)	1	0.34
GG	63 (8.8)	10 (12.2)	1.43 (0.70–2.91)	
Overdominant, n (%)				
AA + GG	416 (58.4)	49 (59.8)	1	0.82
AG	296 (41.6)	33 (40.2)	0.95 (0.59–1.51)	
Allele frequency				
A	1002 (70)	111 (68)	1	0.45
G	422 (30)	53 (32)	0.86 (0.57–1.28)	

Data are presented as n (%) as appropriate.
Abbreviations: ORs, odds ratio.

4. Discussion

In the present hospital-based case-control study, we explored the influence of CTLA4 +49A > G (rs231775) polymorphism on the susceptibility, progression, and outcome of SARS-CoV-2 infection in a sample of Iranian people. To our knowledge, this is the first study to evaluate the association of genetic variation of CTLA-4 with COVID-19 in an Iranian individuals.

The demographic characteristics of study groups revealed that older age and comorbidities like coronary heart disease, hypertension, and diabetes mellitus are more prevalent in COVID-19 cases. Published data have demonstrated that conditions such as cardiovascular diseases, chronic kidney disease, diabetes, and obesity can exacerbate the severity of COVID-19 [38,39]. Baranova et al. suggested hypertension and major depressive disorder might be genetically correlated to increased COVID-19 risk through the induction of inflammatory pathways [40,41].

Although the frequency of the AA genotype in the healthy controls and AG and GG genotypes in COVID-19 patients are more frequent, our findings demonstrated that rs231775 was not associated with the COVID-19 severity and mortality in the Iranian people. Further analysis using logistic regression adjusted for age and gender showed no significant differences among our study groups. Although there were no association studies between rs231775 and COVID-19, there have been numerous studies on the impact of this polymorphism as a host risk factor in various viral infections. Shabbir et al. found a positive association of rs231775 genotype AG with hepatitis C Virus (HCV)-induced Hepatocellular carcinoma (HCC) in the Pakistani population [42]. Wang et al. in a 10-year follow-up, reported a significant association of rs231775 genotype GG with the progression of chronic hepatitis B Virus (HBV) infection to cirrhosis and HCC [43]. The result of the meta-analysis performed by Yu et al. revealed a significant association of rs231775
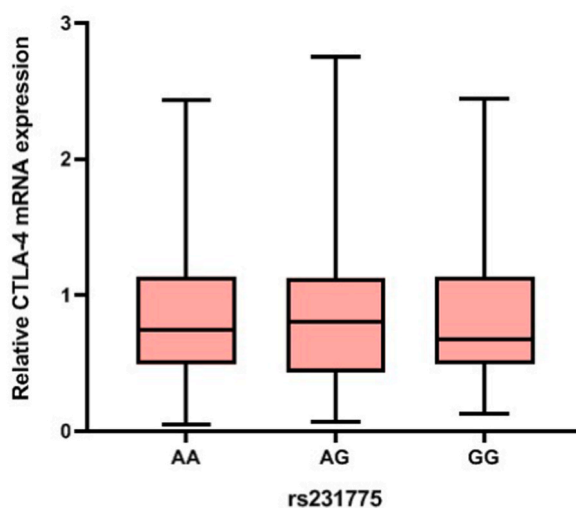

Fig. 1. CTLA-4 mRNA levels in COVID-19 patients according to rs231775 genotypes.
CTLA-4, cytotoxic T-lymphocyte-associated protein 4; mRNA, messenger RNA.

polymorphism in the recessive inheritance model with susceptibility to HBV, especially in East Asian patients [44]. Chen et al. [45] and Gu et al. (24) identified the +49A allele as a risk factor and the +49G allele as a protective factor against HBV infection. Rs231775 Genotype GG contributed to susceptibility to chronic HCV infection in the Brazilian population [46]; however, no significant association was reported in the Chinese population [47]. In addition, no association was detected between the rs231775 polymorphism and the progression of dengue virus (DENV) infection in the study on a Mexican population [48]. Another investigation suggests the +49A allele is linked to a higher risk of parasitic and viral infection [49]. These growing findings highlight the impact of the host's genetic background, with minor allele frequency differences attributed to geographic and ethnic disparities in disease development.

As previously mentioned, +49A > G (rs231775) polymorphism alters the protein structure of CTLA-4 through incomplete glycosylation, resulting in decreased cell surface expression, ligand affinity, and inhibitory activity of this molecule [25]. *In vitro* and *ex vivo* studies of *CTLA-4* showed that the A allele of rs231775 enhanced the ectopic expression and inhibitory effects of CTLA-4 on T cell activation and proliferation compared to the G allele [7,25,50]. Accordingly, the G allele of the CTLA-4 rs231775 can reduce the inhibitory function of CTLA-4 in suppressing activated T cells. Anjos et al. reported that homozygous GG genotypes of rs231775 had a one-third lower surface expression of CTLA-4 on T cells than homozygous AA individuals (25). Considering the previous studies, we hypothesized that different genotypes of rs231775 carrying mutant allele might be significantly associated with the *CTLA*-4 mRNA expression in COVID-19 patients, leading to increased morbidity and mortality. Although our result showed that *CTLA*-4 mRNA levels in COVID-19 patients were significantly lower than in healthy subjects, no significant differences have been found in *CTLA*-4 mRNA expression in COVID-19 patients with different genotypes of rs231775. Thus, this polymorphism does not have a functional impact on the transcriptional activity of *CTLA-4* in our study population.

This study has some limitations that should be considered. In the current study, the relatively small sample size, especially in our stratified analysis, is one of the limitations. Furthermore, another potential limitation is that the patient's mean age was significantly higher than in the controls. In addition, the severity of COVID-19 is determined by multiple factors; this study did not consider the impact of other variables, such as SARS-CoV-2 variants, environmental factors, and additional host genetic variations in our findings. In this respect, further studies are needed to confirm our results in well-matched larger sample sizes and different ethnicities.

5. Conclusion

In conclusion, our findings indicated that SARS-CoV-2 infection is not associated with CTLA-4 +49A > G (rs231775) in the Iranian population. More investigations are needed to show how this genetic variation affects other ethnic groups. Regarding investigating only one polymorphic site of the *CTLA-4* gene in our study, we cannot completely rule out the possibility of other *CTLA-4* gene variants acting as genetic risk factors. So, further studies are recommended to examine other CTLA-4 SNPs and investigate the functional capacity of this gene in COVID-19 patients.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Ensie Sadat Mirsharif: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Abdolrahman Rostamian:** Data curation, Supervision, Validation, Visualization, Writing – review & editing. **Mohammadreza Salehi:** Conceptualization, Investigation, Supervision, Validation, Visualization, Writing – review & editing. **Nayere Askari:** Data curation, Methodology, Supervision, Validation, Visualization, Writing – review & editing. **Tooba Ghazanfari:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23308.

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