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Original Article

The rs568408 variant in the IL-12A gene is associated with risk for COVID-19 in Iraqi patients

Falah Hasan Obayes AL-Khikani[a,b], Zaytoon Abdulridha Alkhafaji[a]*

[a]Department of Microbiology, College of Medicine, University of Babylon, Hilla, Iraq, [b]Department of Microbiology, Al-Shomali General Hospital, Babylon Directorate Health, Babylon, Iraq

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Abstract

Objectives: The objective of the current study was to check the link between potential polymorphism in IL12A rs568408 and the possible risk of COVID-19 in the Iraqi population. **Materials and Methods:** Allele specific-polymerase chain reaction (PCR) technique was carried out for genotyping and detection of IL12A rs568408 gene polymorphism in a case–control study of 125 severe COVID-19 cases and 60 controls. Patients were admitted to either Marjan medical city or Al-Sadeq hospital's COVID-19 wards between January and June 2022 in Iraq. The diagnosis of COVID-19 in each patient was confirmed by severe acute respiratory coronavirus 2-positive reverse transcription-PCR. **Results:** The distribution of both genotyping and allele frequencies of IL-12A rs568408 revealed significant differences between patients and control groups (P = 0.006 and P = 0.001, respectively). The IL12A rs568408 AA and AG variant genotypes were associated with a significantly increased risk of COVID-19 (odds ratio [OR] = 5.19, 95% confidence interval [CI]: 1.13–23.82; P = 0.034) and (OR = 2.39, 95% CI = 1.16–4.94, P = 0.018), respectively, compared with the wild-type GG homozygote. **Conclusion:** These findings indicate that IL12A rs568408 GA/AA variant may contribute to the risk of COVID-19. This study is the first report about the association of IL12A rs568408 with COVID-19.

Keywords: *COVID-19, IL12A, Polymorphism, rs568408*

Introduction

The modern coronavirus disease 2019 (COVID-19) that caused by severe acute respiratory coronavirus 2 (SARS-CoV-2). It was firstly reported in Wuhan, China, in December 2019 [1]. It is the third coronavirus linked to zoonotic origins in the past 18 years, following SARS (2002 and 2003) and Middle East respiratory illness (MERS; 2012 to present) [2]. SARS and MERS stayed geographically limited, whereas COVID-19 has spread worldwide. The disease can present in a variety of ways, ranging from asymptomatic carrier status to death [3]. Patients with functional immunosuppression, such as the elderly and those with prior respiratory or heart diseases, are at a greater risk of infection and unfavorable consequences [4].

Immune cells, including lymphocytes and monocytes/macrophages, express ACE2 considered receptors to SARS-CoV-2 [5], so SARS-CoV-2 may directly infect and damage immune cells. In addition, immune cells can travel around the body. Immune cells exposed to SARS-CoV-2 may let the virus propagate throughout the body. The typical kind of damage caused by SARS-CoV-2 infection also affects the immune system, according to pathological studies using COVID-19 models. Spleen and lymphoid atrophy are connected to considerable cytokine activity, suggesting that SARS-CoV-2 may injure immune cells directly [6,7].

The IL-12A gene encodes a cytokine component that is generated by natural killer and T cells and it has a variety of biological applications. The gene that codes for this cytokine produce a disulfide-linked heterodimer with a 40 kD subunit from the cytokine receptor family and a 35 kD component. IL-12 is necessary for T cell-independent interferon (IFN)-gamma production as well as the development of Th1 and Th2 cells. The transcription activator protein STAT4 regulates the lymphocyte responses to this cytokine [8]. The IL-35 receptor and members of the IL-12 family also share subunits. The IL-35 receptors are made up

**Address for correspondence:*
Prof. Falah Hasan Obayes AL-Khikani,
Department of Microbiology, College of Medicine, University of Babylon, Hilla, Iraq.
E-mail: falahgh38@gmail.com

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of glycoprotein 130 (gp130) and IL12Rb2 (a component of the IL-12 receptor) (IL-27 receptor component) [9].

The correlation between IL-12A rs568408 polymorphism and the risk of chronic HBV infection in the Tunisian population was investigated. Two hundred patients with chronic HBV infection and 200 healthy controls were genotyped using polymerase chain reaction (PCR)-restriction fragment length polymorphism PCR. AA and AG genotypes of IL-12A rs568408 were more represented in the chronic HBV infection group compared to the control group. This study suggested that IL-12A rs568408 contributed to the outcome of chronic HBV infection, it is useful as a predictive and diagnostic biomarker of chronic HBV infection [10].

The functional polymorphism in IL12A that we thought may increase the incidence of COVID-19 in this study. In a high-risk population of Babylon Province, Iraq, genotyping analysis for IL12A rs568408 was conducted in this case–control investigation with 125 severe COVID-19 patients and 60 controls.

Materials and Methods

Subjects of the study

This study enrolled 125 severe COVID-19 patients, 69 (55.2%) females and 56 (44.8%) males aged from 15 to 90 years old. Patients were admitted to either Marjan medical city or Al-Sadeq hospital's COVID-19 wards between January 2022 and June 2022. The diagnosis of COVID-19 in each patient was confirmed by SARS-CoV-2-positive reverse transcription-PCR combined with chest films (X-ray). Patients were determined as severe cases according to the guidelines released by the World Health Organization, patients with clinical signs of pneumonia (fever, cough, and dyspnea) plus one of the following: respiratory rate >30 breaths/min; severe respiratory distress; or SpO2 <90% on room air [11]. As well as 60 healthy persons divided into 32 (53.3%) females and 28 (46.7%) males aged from 18 to 87 years old. Gender and age between severe COVID-19 and control groups were matched. The most common patients in the current study were nonvaccinated 118 (94.4%), whereas vaccinated patients were 7 (5.6%).

All patients with severe COVID-19 symptoms were included in this study. Patients with negative PCR results were excluded as well as patients suffering from asthma or other autoimmune diseases, also patients with a history of cancer were excluded. Healthy groups included persons with no history of COVID-19, without chronic diseases, and without autoimmune diseases.

The current research was approved by the Ethics Committee of Babylon health directorate, Iraq. The date and number of approval was (16744 on 8/1/2022). Before taking the sample, the patients and legal agents were asked to sign the informed consent. Sampling, health, and safety precautions were implemented.

Genotyping

Using the gSYAN DNA extraction kit (Frozen Blood) from Geneaid, USA, and following the manufacturer's instructions, genomic DNA was isolated from blood samples. Using a Nanodrop spectrophotometer (THERMO, USA), which measures DNA content (ng/μL) and checks DNA purity by measuring the absorbance at (260/280 nm), the isolated blood genomic DNA was further processed. Blood samples were examined using an allele-specific-PCR test to identify and genotype the IL12A rs568408 gene polymorphism. The GoTaq® G2 Green Master Mix kit was used to produce the Allele Specific-PCR master mix, and this master mix performed two reactions for each sample under the manufacturer's instructions for both the wild-type and mutant allele types.

In this investigation, the NCBI-SNP data source and Primer1 Allele-Specific PCR primers creation website were used to create allele-specific PCR primers for the IL-K12A rs568408 gene polymorphism. These primers were given by (Scientific Researcher. Co. Ltd. Iraq) [Table 1].

The PCR cycling was set to 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with one cycle at 95°C for 5 min. The last extension lasted 5 min at 72°C.

Biochemical and hematological markers

Using the automated Architect Ci 8200 system, fasting glucose and creatinine levels were measured (Abbott Diagnostics, Lake Forest, IL, USA). Biochemical parameters were detected by reagents, calibrators, and controls depending on the given manufacturer's instructions. Anti-coagulated blood samples from patients are processed for complete blood count through a hematology autoanalyzer (Minami-Ku Kyoto, Japan).

Statistical analysis

The Chi-square test was used to assess differences between cases and controls in the chosen variables, demographic traits, and genotypes of the IL12A-rs568408 variation. Logistic regression analyses were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) in order to determine the relationships between IL12A genotypes and the risk of COVID-19. A goodness-of-fit 2 test was used to assess the Hardy–Weinberg equilibrium by comparing the observed genotype frequencies to those anticipated among the control participants. The Shapiro–Wilk test was employed to determine if the data distribution was normal. Categorical variables were presented as percentages and numbers, and comparisons were made using Fisher's exact test or the Chi-square test. Continuous variables that were not normally distributed were represented as the median (25^{th}–75^{th} interquartile range, [IQR]). Multiple group comparisons were conducted using *post hoc* one-way ANOVA (Duncan's test). The IBM SPSS Statistics version 26 system was used to

Table 1: The IL-K12A rs568408 Allele specific-PCR primers with their sequence and amplicon size

Primer	Sequence (5'-3')	Product size
Wild type Reverse Primer	GATGGGACTATTACATCCACAGA	205 bp
Mutant Reverse Primer	GATGGGACTATTACATCCACAGG	
Common Forward Primer	TGCTTACATGTTTGTTTCCA	

conduct statistical analyses (IBM Corp., Armonk, NY, USA). *P* values under 0.05 were deemed statistically significant.

Results

The present study enrolled 125 patients with COVID-19 and 60 apparently healthy controls. Both patients and controls groups were matched by gender and age are reported as median (IQR) or number (%) and shown in Table 2. The median age of patients was 75 years (62–85), the smallest age was 15 years old, and the biggest one was 90 years old. The median age of controls was 74 years (64–82) and there was no significant difference between patients and controls regarding age ($P = 0.65$). Patients' group included 56 (44.8%) males and 69 (55.2%) females, whereas the control group included 28 (46.6%) males and 32 (53.4%) females and there was no significant difference in the frequency distribution of patients and controls according to gender ($P = 0.87$).

The genotype and allele distribution distributions of IL12A rs568408 in the cases and controls are shown in Table 3. Overall, there was a significant difference in the distribution of IL-12A rs568408 genotypes between cases and control groups ($P = 0.006$).

Logistic regression analysis revealed that the frequencies of the homozygous variant AA and heterozygous variant AG of IL-12A rs568408 were 12% and 36% in COVID-19 cases and 3.3% and 21.7% in healthy controls, respectively. For AA genotype, (OR = 5.19, 95% CI: 1.13–23.82, $P = 0.034$). While AG genotype (OR = 2.39, 95% CI = 1.16–4.94, $P = 0.018$), at IL-12A rs568408 demonstrated a statistically significant risk for COVID-19 in Iraq. The variant rs568408 AG/AA genotypes were associated with a significantly increased risk of COVID-19 (OR = 2.76, 95% CI: 1.40–5.47; $P = 0.003$), compared with the wild-type rs568408 GG.

The combined variant genotypes AG + AA did not further elevate COVID-19 risk that the OR of AA alone (2.76 versus 5.19), compared to the wild-type GG genotype, indicating that A-allele of IL-12A rs568408 behaves as a recessive determinant to COVID-19 risk [Table 3].

The distribution of allelic frequencies of rs568408 in IL-12A showed that the A allele was associated with increased risk of COVID-19, compared to the G allele (OR = 2.59, 95% CI = 1.45–4.63, $P = 0.001$). The frequencies of the A and G alleles were 30% and 70% in COVID-19 patients and 14.17% and 85.83% in control, respectively.

We further evaluated the distribution of IL-12A rs568408 genotypes among some parameters of 125 patients with COVID-19 [Table 4]. There was no significant distribution of rs568408 genotypes regarding urea, creatinine, glucose, WBC, LHD, GPT, GOT, ALP, ESR, and LYM ($P < 0.05$). SpO_2 was very low in GG and AA genotypes ($P = 0.018$). Potassium was very high in AA genotype 5.2 (4.3–5.9) ($P = 0.004$). Total protein was high in AG genotype 62 (57–66) ($P = 0.017$). D-dimer was increased in GG genotype 1458 (585.5–3346) ($P = 0.033$).

No significant differences regarding multiple means comparison of genotype frequency and gender ($P = 0.28$), mortality rate ($P = 0.13$), as well as diabetic patients ($P = 0.57$) in COVID-19 patients.

Table 2: Demographic study of patients with coronavirus disease 2019

Variables	Units	Patients (*n*=125)	Control (*n*=60)	*P*
Age (years)		75 (62-85)	74 (64-82)	0.65
Gender, *n* (%)				0.87
Males		56 (44.8)	28 (46.4)	
Females		69 (55.2)	32 (53.6)	
Creatinine	µmol/L	86 (68-143)	60 (51-73)	<0.001
D-dimer	ng/mL	1200 (537-2928)	127 (82-163)	<0.001
ESR	mm/h	42 (31.5-59.5)	10 (4-13)	<0.001
Glucose	mmol/L	9.5 (5.9-14.4)	4.6 (3.9-5.3)	<0.001
GOT	IU/L	34 (28-45)	15 (12-19)	<0.001
GPT	IU/L	29 (22-41)	21 (14-29)	<0.001
LDH	IU/L	504 (257-788)	120 (87-169)	<0.001
LYM	20%-50%	6.4 (4.2-12	37 (28-43)	<0.001
Potassium (K)	mmol/L	4.2 (3.8-5.1)	4.1 (3.9-4.3)	<0.001
SpO_2	<94	82 (78-88)	>94	<0.001
Total protein	g/L	59 (53.5-65)	69 (61-77)	<0.001
Urea	mmol/L	11.5 (7.1-17.6)	4.5 (3.5-5.8)	<0.001
WBCs	4-11 10^9/L	15,000 (11,570-17,490)	6000 (5200-8300)	<0.001

ESR: Erythrocyte sedimentation rate, GOT: Glutamic oxaloacetic transaminase, GPT: Glutamic pyruvic transaminase, LDH: Lactate dehydrogenase, LYM: Lymphocytes, WBCs: White blood cells

Table 3: Genotyping and allele frequency of interleukin-12A- rs568408 in coronavirus disease 2019 patients and control

Variables	Patients (*n*=125), *n* (%)	Control (*n*=60), *n* (%)	OR (95% CI)	*P*
IL-12A rs568408				
GG	65 (52)	45 (75)	1	1
AA	15 (12)	2 (3.3)	5.19 (1.13-23.82)	0.034
AG	45 (36)	13 (21.7)	2.39 (1.16-4.94)	0.018
AA + AG	60 (48)	15 (25)	2.76 (1.40-5.47)	0.003
P trend				0.006
Allele frequency				
G	175 (70)	103 (85.83)		
A	75 (30)	17 (14.17)	2.59 (1.45-4.63)	0.001
Recessive model				
GG + AG	110 (88)	58 (96.7)		
AA	15 (12)	2 (3.3)	3.95 (0.87-17.88)	0.056
Additive model				
GG	65 (52)	45 (75)		
AA	15 (12)	2 (3.3)	5.19 (1.13-23.82)	0.021
Dominant model				
GG	65 (52)	45 (75)		
AA + AG	60 (48)	15 (25)	2.67 (1.40-5.47)	0.003

OR: Odds ratio, CI: Confidence interval, IL: Interleukin

Discussion

In the current hospital-based case–control study, we looked at the relationship between the Iraqi COVID-19 risk and the IL-12A rs568408 polymorphism. One hundred and twenty-five COVID-19 patients and 60 healthy people of matched age and

Table 4: Distribution of interleukin-12A-rs568408 genotypes among parameters of 125 patients with coronavirus disease 2019

Variables	IL-12A rs568408 genotypes, mean (Duncan's test)			*P* ANOVA
	AA (*n*=15)	AG (*n*=45)	GG (*n*=65)	
SpO_2	80.87[b]	84.91[a]	81.78[b]	0.018
Potassium (K)	5.107[a]	4.40[b]	4.22[b]	0.004
Total protein	55.80[b]	61.29[a]	58.60[b]	0.017
Glucose	8.98	11.97	9.99	0.072
Urea	12.73	13.40	12.97	0.861
Creatinine	174.93	114.49	134.85	0.108
LDH	548.33	602.51	586.82	0.595
D-dimer	2009.92	1661.84	2047.01	0.62
GPT	44.87	46.84	40.11	0.850
GOT	48.80	50.58	47.63	0.758
ALP	112.27	113.44	111.94	0.727
ESR	53.40	49.11	49.94	0.762
WBCs	11.37	15.57	15.26	0.070
LYM	9.59	11.22	8.50	0.748

The different letters in the same raw indicate significant differences (Duncan's test). LDH: Lactate dehydrogenase, GPT: Glutamic pyruvic transaminase, GOT: Glutamic oxaloacetic transaminase, ALP: Alkaline phosphatase, ESR: Erythrocytes sedimentation rate, WBCs: White blood cells, LYM: Lymphocytes, IL: Interleukin

gender made up the sample size. We found that COID-19 risk was determined genomically by SNPs at IL-12A rs568408. Our hypothesis that functional polymorphisms in IL-12 may contribute to the susceptibility of SARS-CoV-2 is supported by our data.

In this investigation, we detected a genetic risk biomarker for predicting COVID-19 susceptibility, the AA genotype at IL-12A rs568408. This genotype was linked to an increased risk of osteosarcoma and esophageal cancer [12,13]. Chen *et al*. demonstrated in 2009 that the genotypes of the IL-12A rs568408 GA/AA variant were significantly associated with an increased risk of cervical cancer. They proposed that rs568408 may interfere with the binding of miRNAs and exonic splicing enhancers, and three SNPs that are in mice-conserved regions may interfere with exonic splicing silencers [14].

In the current study, no significant differences regarding genotype frequency and gender (P = 0.28), mortality (P = 0.13), and diabetic patients (P = 0.57). In another investigation, it was discovered that asthmatic individuals having the A allele at IL-12A rs568408 had worse symptoms [15].

A crucial cytokine in the fight against intracellular infections, interleukin-12, encourages the growth of Th1 cells, cell-mediated cytotoxicity, and IFN-gamma production [16]. Various studies have shown that the two genes that code for IL-12, IL12A, and IL12B, have numerous functional polymorphism sites that may have an impact on the development and spread of lung cancer [17] and tuberculosis [18]. Tan *et al*. found that the IL12A rs568408 variant may be a marker SNP associated with a greater risk of insufficient HBV clearance [19]. However, Ben Selma *et al.* [10] suggested that IL-12A rs568408 and interactions with other genes may affect the course of chronic HBV infection, underlining their potential use as predictive and diagnostic biomarkers. The IL-12 signaling pathway is crucial for HBV infection and may play a role in pathogenesis, according to other evidences [20,21].

In response to microbial stimuli, such as viral infection, dendritic cells and macrophages release IL-12, which interacts with the IL-12 receptor that is primarily expressed by activated T and NK cells. IFN-γ is secreted when IL-15, IL-12, type I IFN, and IL-18 are combined to increase the cytotoxic activity of NK cells. NK cells release IFN-γ, which stimulates macrophages to kill phagocytosed bacteria. Another well-known function of IL-12 is to promote T-helper 1 cell development [22]. A considerably greater amount of IL-12 was produced in patients with mild COVID-19 during the beginning of the acute phase due to SARS-CoV-2 infection than in patients with moderate or severe symptoms and healthy controls. Interestingly, multiple investigations found that patients with severe COVID-19 had much fewer peripheral NK cells than healthy people [23,24], or people who have COVID-19 minor cases [25]. IL-12 induction is necessary to sustain NK cell numbers in the early stages of SARS-CoV-2 infection and this may contribute to the evasion of viral propagation seen in asymptomatic and mildly symptomatic patients [26].

In another investigation, the distributions of IL-12A rs568408 genotypic and allelic frequencies showed significant differences between patients and controls with lung cancer (P = 0.0036 and P = 0.0005, respectively). When compared to the GG genotype, the IL-12A rs568408 AA genotype was specifically linked to a substantially increased risk of lung cancer (OR = 2.41, 95% CI = 1.36–4.29, P = 0.0021) [27].

Another study mentioned that IL-12A rs568408 GA (P = 0.035), and rs568408 GG/AA (P = 0.034) were associated with an increased chance for the development of anti-hepatitis B virus surface antigen in hemodialysis patients. Patients bearing rs568408 AA had a 10.9-fold or 8.9-fold chance to develop antibodies compared with those carrying any other genotype (P = 0.005) or those who had both wild-type rs568408 GG [28].

In a sample of the southeast Iranian population, research revealed that IL12A rs568408, polymorphism was not a significant genetic determinant for resistance or susceptibility to pulmonary tuberculosis. In patients and controls, the rates of the GA and AA genotypes of the IL12A rs568408 variation were 38.5%, 1.7%, and 37.3%, 1.1%, respectively [18].

The genotype and allele frequencies of IL-12A rs568408 in asthma were significantly different between patients and controls (P = 0.001). The AC genotype of rs3212227 was associated with a significantly decreased risk of having asthma when compared to the AA genotype (P = 0.036). Individuals with the coupled genotypes (rs568408 AG and rs3212227 AC/CC) had a 2.05-fold increased risk of having asthma compared to those with all other genotypes (P = 0.001). When rs568408 GG and rs3212227 AC/CC were combined, the probability of

developing asthma was significantly lower than when rs568408 GG and rs3212227AA were combined ($P = 0.009$) [29].

In the current study, the genotype AA associated with increased potassium (K^+), decreased in SpO_2, decreased in a total protein of severe patients with COVIV-19. In this study, the mean of SpO_2 in patients with genotype AA was 80.87%. Normal SpO_2 levels in humans are 97%~100%. If the level is below 90%, it is considered low and called hypoxemia. SpO_2 levels below 80% may compromise organ function, such as the brain and heart. Continued low oxygen levels may lead to respiratory or cardiac arrest [30]. Reasons why hypoproteinemia develops in COVID-19 remain unclear. Anorexia/vomiting and diarrhea, in order, are reported in 25.8% and 29.8% of all COVID-19 patients [31]. Inadequate food supply secondary to these symptoms may direct the body toward protein breakdown for energy production. As well as SARS-CoV-2 is associated with a hypercatabolic state that entails excessive protein loss [32]. A prospective cohort study showed that critically ill patients in the medical ICU with abnormal K^+ levels had a higher incidence of ICU mortality than patients with normal K^+ levels [33].

The main limitation of this work was the small sample size, which made it more likely that false-positive or false-negative results would result from weaker statistical analysis. As a result, the study's statistical power is constrained, particularly for subgroup and interaction analyses. Therefore, to further understand the effect of IL12 SNPs on COVID-19 susceptibility, extensive prospective studies with ethnically varied inhabitants are required. Furthermore, we were unable to determine the IL-12 expression levels in the participants under investigation, which restricted the further examination of the genotype-phenotype connection and any potential relationships between phenotypic traits and prognosis and outcomes.

Conclusion

The results of the current study suggested that the COVID-19 etiology may be determined by the A allele in IL-12A rs568408. We think that this discovery may help us understand how the IL-12A gene affects COVID-19 regulation and prediction accuracy. It is necessary to conduct further research on the phenotypes of the A and AG alleles at IL-12A rs568408. The AA IL-12A rs568408 genotype may be a widespread genetic biomarker for COVID-19 in different COVID-19 populations, according to these data.

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Conflicts of interest

There are no conflicts of interest.

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