



Journal of Acute Disease

Original Article



jadweb.org

doi: 10.4103/jad.jad_80_24

Impact Factor® 0.5

Changes in the gene expression of activation-induced cytidine deaminase in peripheral blood mononuclear cells in COVID-19 patients

Nioosha Ahmadi¹, Hossein Lajmire¹, Saeedeh Ebrahimi^{1,2}, Hadi Razavi Nikoo^{3,4}, Mehrdad Farrokhnia⁵, Elham Heidari⁶, Elham Mousavi^{1,2}✉

¹Department of Medical Microbiology (Bacteriology and Virology), Afzalipour Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran

²Medical Mycology and Bacteriology Research Center, Kerman University of Medical Sciences, Kerman, Iran

³Infectious Disease Research Center, Golestan University of Medical Sciences, Gorgan, Iran

⁴Department of Microbiology, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran

⁵Infectious and Tropical Research Center, Kerman University of Medical Sciences, Kerman, Iran

⁶Department of Anatomy, School of Medicine, Iranshahr University of Medical Sciences, Iranshahr, Iran

ABSTRACT

Objective: To assess the changes in gene expression of activation-induced cytidine deaminase (*AID*) in COVID-19 patients and its relationship with the presence of autoantibodies in those ones.

Methods: This retrospective study was carried out among COVID-19 patients' serum and peripheral blood mononuclear cells that were archived at the main COVID-19 referral centers in Iran from January to March 2022. The titer of autoantibodies in the serum of healthy and COVID-19 subjects was investigated by serological tests. Then, the *AID* gene expression in peripheral blood mononuclear cells in two groups was evaluated using the real-time polymerase chain reaction method.

Results: 100 patients were included. In total, 8% and 1% of patients had a significant titer of anti-citrullinated protein antibody and antinuclear antibody autoantibodies, respectively, and those suffered from at least one comorbidity. No patient was positive for antiphospholipid autoantibody. Moreover, no significant changes were observed in the titer of autoantibodies between healthy subjects and patients. Furthermore, the increase in the expression of the *AID* gene was not significant in two groups.

Conclusions: The presence of autoantibodies in COVID-19 patients might be linked to the previous underlying disease.

KEYWORDS: SARS-CoV-2; Activation-induced cytidine

deaminase; Anti-nuclear antibody; Anti-phospholipid antibody; Anti-cyclic citrullinated peptide antibody

Significance

This study investigates the relationship between autoantibodies and the expression of the activation-induced cytidine deaminase gene in COVID-19 patients. The findings reveal that while a small percentage of patients exhibited specific autoantibodies, there were no significant differences in antibody titers or activation-induced cytidine deaminase gene expression compared to healthy controls. These results suggest that the presence of autoantibodies in COVID-19 may be more closely associated with underlying health conditions rather than direct effects of the virus.

✉To whom correspondence may be addressed. E-mail: emosavi94@yahoo.com

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How to cite this article: Ahmadi N, Lajmire H, Ebrahimi S, Nikoo HR, Farrokhnia M, Heidari E, et al. Changes in the gene expression of activation-induced cytidine deaminase in peripheral blood mononuclear cells in COVID-19 patients. J Acute Dis 2024; 13(5): 173-178.

Article history: Received 1 August 2024; Revision 1 August 2024; Accepted 25 November 2024; Available online 29 November 2024

1. Introduction

COVID-19 caused by SARS-COV-2 leads to a range of mild cold-like symptoms to severe pneumonia, acute respiratory syndrome, and even death in individuals[1,2]. Moreover, some clinical manifestations similar to autoimmune diseases such as polyarthritis, muscle pain, chronic fatigue, petechia, myocarditis, and thrombosis have been also observed in COVID-19 patients[3,4]. Due to these complications, SARS-COV-2 seems like some viral infections including Epstein-Barr virus (EBV), cytomegalovirus, human immunodeficiency virus (HIV), and human T-lymphotropic virus 1 might have a significant role in the cause of autoimmune diseases[5-8].

Besides, patients with COVID-19 show an imbalance of the immune system including cytokine storm, and hyperactivation of immune cells[9,10]. Furthermore, the presence of autoantibodies such as antinuclear antibodies, Antiphospholipid antibodies, and anti-citrullinated protein antibodies have been reported in several studies[11,12]. These autoantibodies have been attributed to the disease severity, the triggering of chronic and persistent inflammation, long COVID-19 symptoms, and even death[13,14]. Although several studies have been conducted on the cause of the emergence of autoimmune disorders in COVID-19 patients, the mechanism is still ambiguous[15,16].

Activation-induced cytidine deaminase (*AID*) is one of the host's immune system enzymes which is expressed in B cells stimulated with primary antigen in lymphoid organs and produces the antibodies with high binding affinity against pathogens[17]. The main place of expression of this enzyme is in B cells of germinal centers, in lymphoid tissues[17]. However, studies show that in some cases, this enzyme is expressed in places outside of lymphoid tissues and can lead to diseases such as cancer, chronic inflammation and autoimmune diseases[18-21]. In many inflammatory diseases such as asthma, allergic rhinitis and irritable bowel syndrome, increased expression of *AID* has been observed in nasal mucosa cells and gastrointestinal cells[21,22]. In addition, evidence shows that some viral pathogens such as HCV, EBV, and HIV can increase the expression of this enzyme in the peripheral mononuclear blood cells (PBMCs) and liver tissues[23-25]. This retrospective study investigated the presence of prevalent autoantibodies measured in the serum of COVID-19 patients such as antinuclear antibody (ANA), antiphospholipid antibody (APA) and anti-citrullinated protein antibody (ACCP) in comparison to a healthy people group. Then, the gene expression of *AID* in PBMCs of both patients and healthy groups was assessed to clarify the probable role of SARS-COV-2 in *AID* gene expression and the presence of autoantibodies.

2. Patients and methods

2.1. Serum and PBMCs samples

The samples were collected from COVID-19 patients' serum and PBMCs that were archived at the main COVID-19 referral centers in Iran from January to March 2022, the period after the pandemic peak, when nearly all people got vaccinated against SARS-COV-2 in Iran. The demographic and clinical information was retrieved from the patient's electronic medical record. The healthy subjects were the individuals who were similar at the same age and sex group with included patients. The informed consent from the patients and permission from the Ethics Committee of Kerman University of Medical Sciences (Ethical code IR.KMU.AH.REC.1402.018.) was obtained.

2.2. Measurement of autoantibodies in serum

The autoantibodies determination was performed using chemiluminescent microparticle immunoassay (ARCHITECT i2000SR, USA) method with the ANA (Generic Assays, Berlin, Germany), APA (AESKU, Germany) and ACCP (DiaMetra, Italy) Kits according to the manufacturer's protocols in the collected samples. The measurable range of this assay for autoantibodies was 0.05-250 IU/mL. According to the instruction of the kit, if the autoantibody titer in the serum of patients is higher than 10 IU/mL, it is reported as positive, on the other hand, the sample with a titer lower than 10 IU/mL is reported as a negative sample.

2.3. Gene expression of *AID* in PBMCs cells

Trizol reagent (YTzol Pure RNA, Iran) was used to extract total cellular RNA from PBMCs samples. The quantity and purity of RNA were determined by measuring the absorbance at 260 nm and the A260/A280 ratio in a UV spectrophotometer (Nanodrop Inc, Wilmington, DE USA). The cDNA was synthesized by cDNA Synthesis Kit (Yektataghiz, Iran) based on the manufacture

Table 1. The sequences of primers in this study.

Gene	Primer
<i>AID</i>	F: 5'- CCGACTTTCTTCGAGGGAAC- 3' R: 5'- GGTTCCTACAAAAGTATTCCAGCA- 3'
<i>HPRT</i>	F: 5'- CCCTGGCGTCGTGATTAGTG- 3' R: 5'- TCGAGCAAGACGTTTCAGTCC- 3'

AID: activation-induced cytidine deaminase; *HPRT*: hypoxanthine guanine phosphoribosyl transferase.

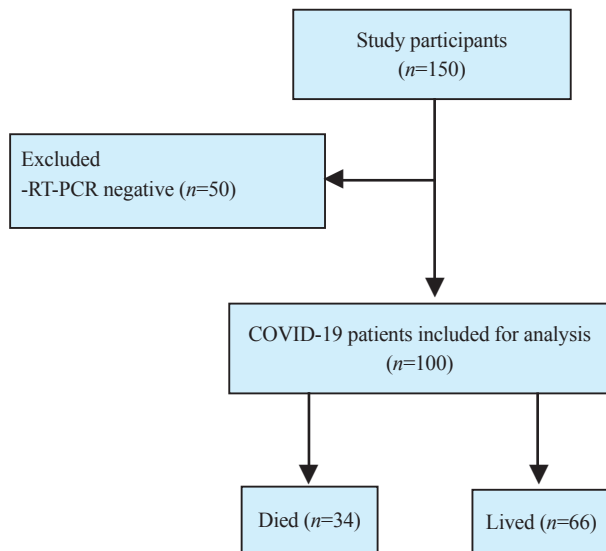


Figure 1. The study flowchart.

recommendation. Then, the real-time PCR reaction was prepared by SYBR Green qPCR master mix (Amplicon, Denmark), containing 5 μ L of Master Mix, 0.5 μ L forward primer, 0.5 μ L reverse primer, 1 μ L template DNA, 3 μ L DEPC water in a temperature program as follows: 95 $^{\circ}$ C (15 mins) $\rightarrow$ 95 $^{\circ}$ C for (30 s) $\rightarrow$ 53 $^{\circ}$ C (30 s) $\rightarrow$ 72 $^{\circ}$ C (30 s). The amplification and data capture were performed using SYBR Green Chemistry in Rotor-Gene 6000 (Applied Biosystems, Germany). To normalize the value of the target's mRNA, the hypoxanthine guanine phosphoribosyl transferase gene was measured in all samples and the obtained data was analyzed using the $2^{-\Delta\Delta C_t}$ method[26]. Table 1 shows the primer sequence used in this study.

2.4. Statistical analysis

All data in this study are presented as means $\pm$ standard deviation. Graph Pad Prism v.8.0.1 software and SPSS 26 were used for statistical analysis. *t* test were used for comparison of the autoantibodies value and cycle threshold mean between two groups of patients and the control.

3. Results

3.1. Demographics and clinical profile of participants

In the present study, the samples from one hundred COVID-19 patients and fifty healthy individuals were assessed. The study flowchart was demonstrated in Figure 1. In total, 55 (55.0%) males with an average age of 65.1 years and 48 (45.0%)

females with an average age of 63.2 years were grouped as COVID-19 patients. An equal number of men and women with an average age of 58.5 years participated as a healthy group. Regarding the hospitalization ward of patients, 45.0% and 55.0% of subjects were hospitalized in the ICU and infectious ward respectively. The most common Comorbidities among included patients were circulatory system diseases (51.1%), metabolic disorders (50.0%), respiratory system diseases (41.3%), and genitourinary system disorders (25.0%). A total of 34% of patients included in this study had died of COVID-19.

3.2. Presence of ANA, APA, and ACCP autoantibodies in serum

According to the results obtained from the ELISA, 8 (8%) patients had significant titers of antibodies against cyclic ACCP. ANA was detected in the serum of one patient (1%), and none of the patients were positive for APA (0%). Moreover, there wasn't a notable titer of these types of autoantibodies in healthy individuals. The results are summarized in Table 2 (ANA, $P=0.426$; APA, $P=0.721$; ACCP, $P=0.329$). Additionally, the demographic and clinical information of the patients were shown in Table 3.

3.3. Comparison of autoantibody titer value in two groups

After measuring autoantibody titer in the serum of both COVID-19 patients and healthy individuals, the statistical analysis showed that the titer of all autoantibodies in COVID-19 patients is higher than in healthy ones. However, this increase was not statistically significant (ANA, $P=0.425$; APA, $P=0.720$; ACCP, $P=0.328$) (Figure 2).

3.4. Comparison of AID gene expression in two groups

As Figure 3 shows the mRNA expression levels of *AID* in the patients' group increased compared to the healthy ones. Although this increase was not statistically significant (fold change=1.847, $P=0.234$).

Table 2. Frequency of autoantibodies in serum of both patient and healthy groups.

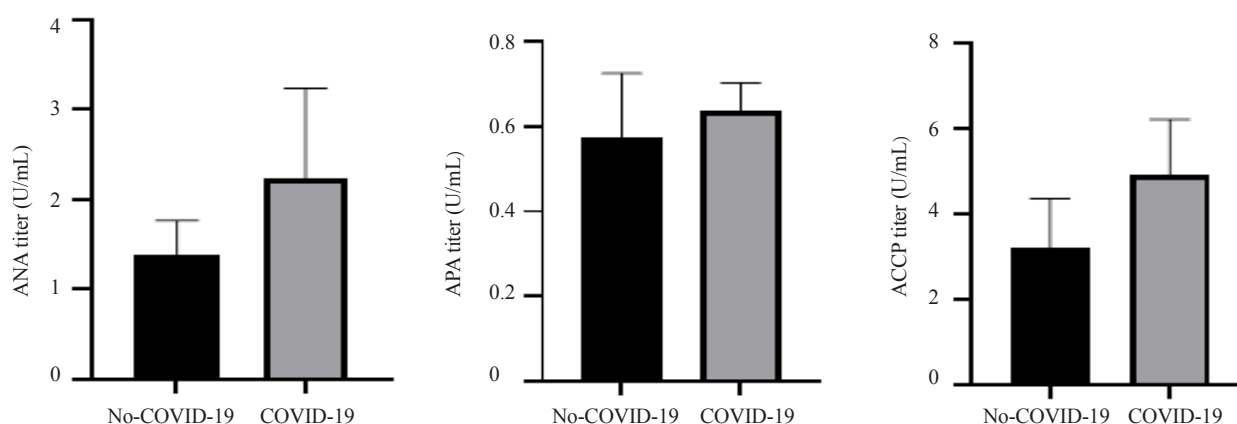
Autoantibodies	Patient group	Normal group
ANA	1	0
APA	0	0
ACCP	8	0

ANA: antinuclear antibody; APA: antiphospholipid antibody; ACCP: anti-citrullinated protein antibody.

Table 3. Clinical profile of COVID-19 patients with positive autoantibodies.

Patients code	Sex	Age (years old)	Hospitalization ward	Autoantibodies (IU/mL)	Comorbidities	Disease outcome
P3	Male	77	Infectious	ACCP 12	Ischemic heart disease, hypertension, diabetes, prostatic hyperplasia	Deceased
P27	Male	70	ICU	ACCP 13	Ischemic heart disease, hypothyroid	Recovered
P32	Female	59	Infectious	ACCP 105	Diabetes, asthma, rheumatoid arthritis, colon cancer, ecthyma	Recovered
P42	Male	51	Infectious	ACCP 12	Clinically isolated syndrome	Recovered
P43	Male	40	Infectious	ACCP 11	Clinically isolated syndrome	Recovered
P79	Female	70	ICU	ACCP 10	Sepsis, breast cancer	Deceased
P80	Female	74	ICU	ANA 82	Hypertension, diabetes, hyperthyroid, coronary artery bypass graft surgery	Deceased
P82	Female	67	ICU	ACCP 14	Hypertension, depression	Recovered
P84	Male	74	ICU	ACCP 11	Sepsis, HBV infection	Deceased

ICU: intensive care unit; HBV: hepatitis B virus.

**Figure 2.** Comparison of autoantibodies' titer in the serum of COVID-19 patients and healthy subjects. a) ANA; b) APA; c) ACCP.

4. Discussion

In the present study, autoantibodies were detected in the serum of a small percentage of COVID-19 patients. However, in previous studies, the presence of these autoantibodies has been reported, from 15% to 57%[11,27,28]. Several reasons can explain these discrepancies. Firstly, in some studies, the titer of autoantibodies has not been assessed and thus a false positive result might have been reported. To explain vividly, for instance, ANA autoantibody with a titer of 1:80 is found in the serum of nearly 14% of healthy people[29-31]. Therefore, it is possible that COVID-19 patients had a measurable titer of this type of autoantibody in their serum before

COVID-19 infection and without any underlying disease. Moreover, in a study conducted by Mihailo *et al.*, although the frequency of ANA autoantibody in COVID-19 patients was reported about 20%, the presence of this autoantibody was not related to the severity of the disease[32]. Another reason for the high titer of autoantibodies in COVID-19 patients might be related to the underlying illnesses. As Table 3 shows in the present study, patients with the detectable autoantibody titer, suffered from at least one comorbidity such as prostatic hyperplasia, chronic heart disease, colon cancer, diabetes, rheumatoid arthritis, chronic respiratory system disease, and asthma. Therefore, the underlying diseases might be the cause of the presence of these autoantibodies in COVID-19 patient's serum.

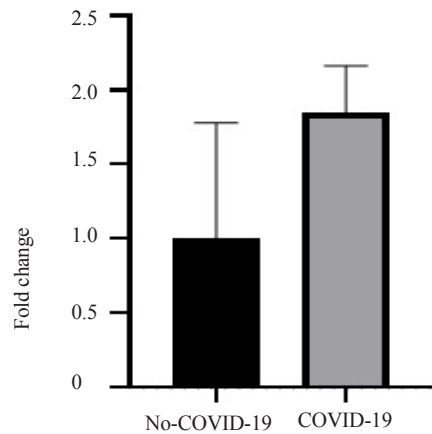


Figure 3. Comparison of *AID* gene expression in two groups of COVID-19 patients and healthy people.

As mentioned in the results, no increase in the expression of the *AID* gene was observed in peripheral blood cells of patients infected by SARS-CoV-2. However, in some viral infections such as HCV, HBV, HIV and EBV, the excessive expression of this enzyme outside the lymphoid tissues such as PBMCs and liver cells, was proved[23-25]. Considering that there is little evidence about the infection of PBMCs by SARS-CoV-2, it's not far-fetched that no changes were observed in the expression of this enzyme in PBMCs.

Overall, according to the findings of this study, there was no significant increase in the level of measured autoantibodies in the blood of patients with COVID-19. Furthermore, those patients with measurable autoantibodies suffered from at least one underlying disease. Therefore, more investigation needs to identify the cause of the presence of autoantibodies in the previous studies.

Conflict of interest statement

The authors report no conflict of interest.

Funding

There was no extramural funding received for conducting this study.

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

AN, LH, ES, R-NH,HE, and FM performed experiments, collected the data, and wrote the manuscript; ME is the supervisor; ME analyzed the data, evaluated and interpreted the results; All authors approved the current version of the manuscript.

Acknowledgement

The Ethical Committee of Kerman University of Medical Sciences (IR.KMU.AH.REC.1402.018 with grant number 401000648) approved the study.

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Edited by Liu JJ