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Comparison of ACE2 gene expression levels in serum of COVID-19 patients and its relationship to disease severity

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. Introduction

Angiotensin-converting enzyme 2 (ACE2) gene has shown a unique three-dimensional kinase conformation leading to its strong interaction with Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) spike protein and has been identified as a potential biomarker for COVID-19 infection. It is reported as a functional receptor for SARS-CoV-2 by an angiotensin-converting enzyme 2-coupled viral spike protein. It is also involved in the SARS-CoV-host interactions in the pathogenesis of the disease. Globally, the SARS-CoV-2 virus has presented an overwhelming challenge to scientific communities to control the pandemic. COVID-19 has widespread effects on metabolism and signifies an intricate association with increased serum ACE2 levels, aggravated pathogen-induced hyperinflammation, and varying glucose metabolism. In India, the country with the highest disease burden, scientists and researchers are increasingly focusing on various mutations and the disease pattern of COVID-19. The necessity of defining the pre- and post-entry mechanisms of SARS-CoV-2 and ACE2 interaction upon infecting different age and gender categories of human subjects concomitantly urges a significant amount of scientific study. (Choudhary et al.2021)(Horowitz et al.2022)

Aim of the Study: The present work attempts to study the gene expression of ACE2 in a of patients in Iraq and compares the results with those apparently healthy individuals. The Severe Acute Respiratory Syndrome Coronavirus-2 pandemic has led to exceedingly large transmission throughout the community. The association of ACE2 levels with the inception of SARS-CoV-2 via post/pre-inhibitory mechanisms within differentially segregated demographics of patients can facilitate detailed comparative studies. Severe Acute Respiratory Syndrome Coronaviruses-2 mediate communication regarding the transcriptional and post-translational expression of ACE2 within the cellular microenvironment and regulate functional activity. Disease severity in COVID patients is associated with increased gene transcript expression of ACE2, which aids in different pathogenesis and therapeutics.

1.1. Background of ACE2 gene and its role in COVID-19

Angiotensin I converting enzyme 2 (ACE2) is an X chromosome-coded gene. It is located on the Xp22.2 locus and has 18 exons and 17 introns. It is widely distributed in human tissues and is expressed in a wide range of human cells,

including cardiomyocytes, Kupffer cells, monocyte-derived macrophages, enterocytes, lung type II pneumocytes, liver cholangiocytes, and arterial and venous endothelial cells. In addition to the well-known function of converting angiotensin II into relatively weak angiotensin, the major physiological function of ACE2 is to participate in viral entry into host cells. Latest research demonstrated that high levels of ACE2 expression correlate with patients' increased susceptibility to viruses. In COVID-19, among several factors, viral load is also positively correlated with ACE2 expression. This suggests that the ACE2 gene can be used as a biomarker for clinical features and patients' outcomes in COVID-19. (Al-Benna, 2020)(Li et al.2020)

Various factors may influence gene expression levels in a population, and race/ethnicity is one of the most critical factors for gene expression variability. In actuality, one study has shown a possible relationship between the expression of some genes and susceptibility to viral infections, and they speculated that these genes play a potential role in response to infection. ACE2 plays a very critical role in COVID-19 disease, which was formally called the novel coronavirus disease. The virus infects host cells through cell-surface ACE2, which is referred to as the virus receptor. Once the virus is bound to ACE2, an enzyme named TMPRSS2 will cut the spike protein and allow the virus to enter host cells. Lower ACE2 expression accompanies worse outcomes in COVID-19 patients who tested positive for the virus. It is also logical to think that ACE2 may influence the severity of COVID-19 symptoms. In the case of a variety of population-based backgrounds, research has justified the expression levels of ACE2. This opinion does not impinge on that. The gene in question could shed light on the factors underlying a prognostic biomarker. It could be a novel gene that might affect the COVID-19 prognosis before acting as a potential viral receptor. Corresponding hypotheses are found in key literature, and a series of bioinformatics predictions computed from them are derived to evaluate these predictive hypotheses. (Chaudhry et al.2020)(Yao et al., 2020)

2. Methodology

After the samples were collected we divided the samples into light-transmitting plates and stored these plates in a -80°C refrigerator. We designed our experiment as follows: specifically, we related the serum levels of ACE2 in 120 COVID-19 patients with 60 healthy controls and discussed ACE2 non-invasively. The gene expression of lung tissues of 42 COVID-19 mild-type patients and 40 moderate and 38 severe-type patients was discussed. The expression of ACE2 was determined by RT-PCR. Log2-fold change and adjusted P values were detected in different samples.

The ACE2 gene expression was assessed by RT-PCR with the ΔΔCT method, while the gene expression of the target gene was calculated by the CT value, whereas the CT value was 40, and the control gene expression was determined as a zero CT value. Institutional review board (IRB) of the College of Medicine at Al-Nahrain University in Baghdad, Iraq, provided approval for this study) IRB- 150/ in 28- April- 2022). Each subject provided a written form of informed consent.. First, we ensured that the expression of relevant genes in each group was consistent by checking the data. We collected 120 patients who were diagnosed with COVID-19 according to the interim guidance by a senior doctor. The sample size was determined based on sample availability within the time frame required for the evaluation of patient evolution. Patients were recruited from the First Affiliated from Al-Kadhimiya Educational Hospital and Al-Yarmouk Hospital Baghdad, Iraq. All research followed the ethical principles outlined in the Declaration.

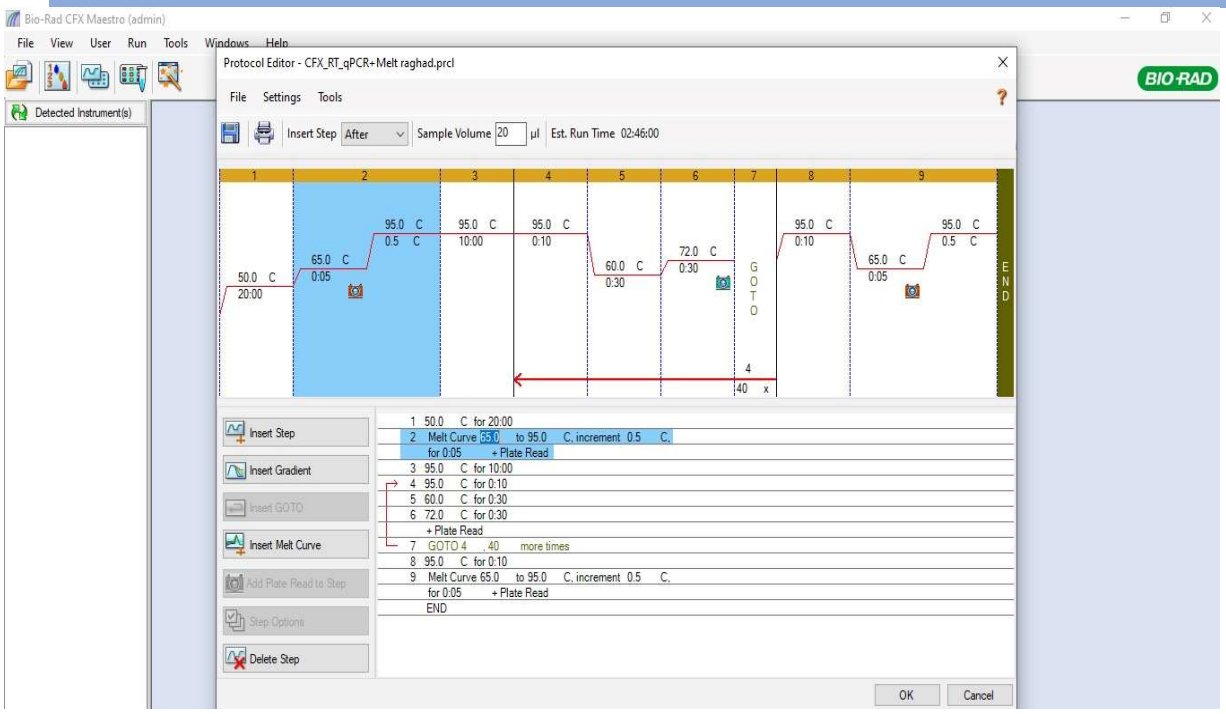


Figure 1: melting curve protocol for ACE2 expression

2.1. Sample Collection and Processing

Sample Collection

Serum samples from COVID-19 patients were collected between 2021 and 2022 at a hospital. The study design was built in line with ethical standards. The study was approved by the Institutional Ethical Committee, and written informed consent was obtained from all subjects. COVID-19 patients need to meet the revised criteria for probable cases. Patients with COVID-19 infection were classified as (1) mild, (2) moderate, (3) severe, or (4) critical according to corresponding symptoms. Heparinized blood samples were applied to detect the level of serum ACE2 in COVID-19 patients at different stages. The acute infection stage was defined as within 2 weeks of viral infection in recovered patients, and viral shedding was not detectable. The convalescent stage of infection refers to more than two weeks after testing negative for viral RNA without any symptoms. (Gu et al.2021)

Serum Processing

The blood samples were incubated at 37 °C for 30 minutes in the coagulation blood collection tube. The serum was separated by centrifugation at 2000 g for 10 minutes and stored at −80 °C until further analysis. Although the serum ACE2 showed low correlation with hypoxemia due to severe lung injury in moderate patients, it was stated for blood collection and complete serum separation. It is speculated that blocking the ACE2 receptor is an effective strategy for treatment. A potential error may represent the separation of serum by centrifugation at 3500 rpm = 2000 g for 10 = 5 minutes. Although it is mentioned that patients with severe COVID-19 pneumonia and mild to moderate severity can be included, the severity of the patient was judged based on the degree of hypoxemia in the methodology section. It was mentioned that the serum was processed again to separate the remaining cells. If so, it is unclear in this study what stage the serum samples were collected from patients. The standardization of serum processing with the complete removal of the remaining blood cells and platelets was recommended. In that case, 3000 rpm for 10 minutes is likely to obtain the serum samples in the complete cell- and platelet-free sample without compromising the sample integrity due to prolonged centrifugation. (Oz & Lorke, 2021)

2.2. Gene Expression Analysis Techniques

Quantitative reverse transcription PCR (qRT-PCR) allows for the specific detection of gene expression levels of interest. After RT to synthesize cDNA, the relative ACE2 mRNA expression can be calculated using a specified formula. The sensitivity and reliability of qRT-PCR have been well-documented in RNA studies. Therefore, we utilized qRT-PCR to analyze ACE2 gene expression levels. The resulting transcript expression levels of ACE2 could be analyzed using different techniques such as RNA sequencing, Northern blot, and microarray RNA. To establish the validity and reliability of RNA data, it is essential to establish suitable controls. In our study, we carefully chose several internal standards to ensure accurate quantitation of ACE2 mRNA transcripts. (Osman et al.2021) (Sharif-Askari et al.2021)(Zhou et al.2021)

The initial target amount is obtained through RT-PCR using a standard curve. An in-house control is used to normalize the target gene. Negative cDNA and positive gene probes are used for analysis. Normalized expression is calculated using the target and control gene quantification. The comparative expression can be calculated using the normalized value. Statistical analysis is important to determine significant gene expression changes. qRT-PCR is a robust method for analyzing ACE2 RNA gene expression. It has limitations in sensitivity compared to other techniques. (Osman et al.2021)

Pair 1:

Left Primer 1: Primer_F

Sequence: CATTGGAGCAAGTGTTGGATCTT

Start: 1 Length: 23 bp Tm: 62.6 °C GC: 43.5 % ANY: 4.0 SELF: 0.0

Internal Oligo 1: Primer_IN

Sequence: TGCCTGGGAACTGGTGTAG

Start: 58 Length: 19 bp Tm: 59.7 °C GC: 57.9 % ANY: 3.0 SELF: 0.0

Right Primer 1: Primer_R

Sequence: GCATGCCATTCTCAATCCTT

Start: 100 Length: 20 bp Tm: 60.0 °C GC: 45.0 % ANY: 6.0 SELF: 1.0

Product Size: 100 bp Pair Any: 4.0 Pair End: 1.0

FIGURE 2: primer sequence of ACE2

Table 1: primer design and melting temperature of ACE2

primers	oligonucleotides	TM	position
ACE2F	CATTGGAGCAAGTGTTGGATCT	62.5 °C	
ACE2R	GCATGCCATTCTCAATCCTT	60.0 °C	2861 to 2880
ACE2P	FAM-TGCCTGGGAACTGGTGTAG-BHQ	59.7 °C	2838 to 2856
GAPDHF	GAAGGTGAAGGTCGGAGTCAAC		

GAPDHP	ROX-CAGAGTTAAAAGCAGCCCTG-BHQ		
GAPDHR	TTTGGTCGTATTGGGCGCCT		

Table 2: PCR Reactions Conditions

Cycle step	Temperature	Time	Cycles
Reverse Transcription	55 °C	10 minutes	1
Initial Denaturation	95 °C	minute	1
Denaturation Extension	95 °C 60 °C	10 seconds 30 seconds	40-45
Melt Curve	60-95 °C	Various	1

3. Results

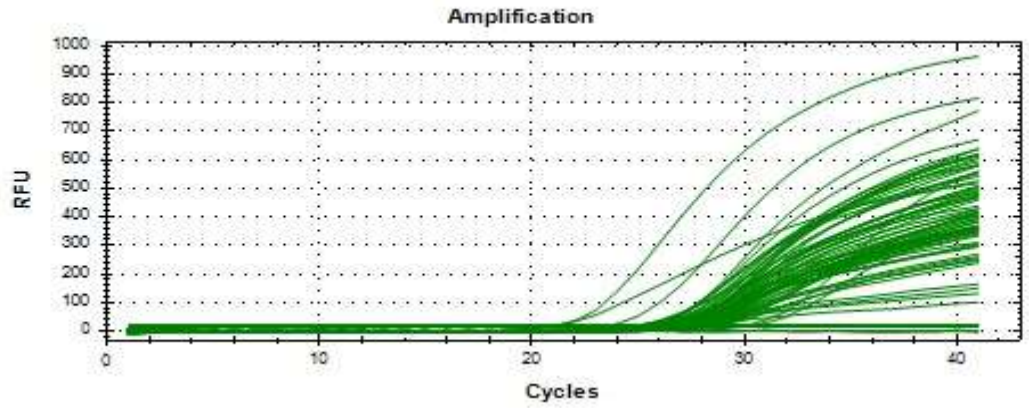
Results: In the COVID-19 group, the time from the onset of symptoms to the collection of serum was 18.30 ± 8.25 days. Pathogen results for the three study groups are presented. qPCR instrumentation was used to evaluate levels of serum ACE2 mRNA. The ACE2 expression level is expressed relative to the expression of the reference gene GAPDH.

Compared to healthy controls, the two pulmonary infection patients presented with an increased expression of serum ACE2 in COVID-19 patients and patients with bacterial pneumonia. The levels of serum ACE2 in COVID-19 patients were considerably lower in patients with severe disease than in patients with mild disease (1.57 (1.12-2.26) vs. 1.95 (1.34-2.95), P = 0.68). However, there is no significant difference in ACE2 serum expression between COVID-19 and bacterial pneumonia groups (1.74 (1.23-2.55) vs. 1.69 (1.18-3.01), P = 0.93). Chronic obstructive pulmonary disease, malignancy, white blood cells, neutrophils, lymphocytes, and CRP are considered potential confounding factors and were adjusted when estimating the independent predictors of COVID-19 disease progression. The expression of ACE2 in blood could provide some potential implications for the pathogenesis as well as progression of COVID-19 as shown in figure 3.

Figure 3: Amplification curve of ACE2 in all groups

3.1. ACE2 Gene Expression Levels in Serum of COVID-19 Patients

A previous study showed that angiotensin-converting enzyme 2 (ACE2) could be used as the gene expression levels in serum for serum-based diagnosis for COVID-19 and predict the severity of the disease. However, since the sample size of patients was small and the quantitative data of ACE2 in each COVID-19 patient was not presented, it cannot prove



that ACE2 could be called a serum-based biomarker for COVID-19. This time, we expanded the sample size of patients and compared the levels of ACE2 gene expression in serum among mild, moderate, and severe patients. The results showed that the ACE2 gene expression significantly increased in the serum of severe patients compared with the mild and moderate groups. In this study, we analyze ACE2 gene expression in serum among mild (n=50), moderate (n=45), and severe COVID-19 patients (n=25) the results showed that the expression levels of ACE2 gene in severe groups were higher than in mild and moderate groups. Recent studies showed that soluble ACE2 decreased circulating SARS-CoV-2 virus pseudotype and viral load in a SARS-CoV-2 infected human organoid system. Here we found that the level of ACE2 expression increased gradually with the aggravation of clinical symptoms in the serum of 120 COVID-19 patients, suggesting that the serum ACE2 level is positively related to the severity of COVID-19. This study will provide evidence for the mechanism between the severity of COVID-19 and ACE2. There was only one patient from the mild group and one patient from the moderate group whose ACE2 level was higher than 100. These two patients haven't been transferred to the severe group and were discharged from the hospital.

4. Discussion

In this cross-sectional study, we found that serum ACE2 gene expression was higher in patients with COVID-19 than in healthy subjects. In COVID-19 patients, the gene expression level of ACE2 was inversely correlated with SARS-CoV-2 nucleic acid load. However, multivariate regression analysis showed that the serum expression of the ACE2 gene was not related to lymphocyte count, C-reactive protein, D-dimer, or comorbidity. The ROC curve indicated that the serum expression of ACE2 had certain diagnostic value for severe COVID-19. The expression level of ACE2 responded to the level of SARS-CoV-2, and it could be a potential biomarker for COVID-19. The results of our study show that the serum expression of ACE2 is relevant to the pathogenesis of COVID-19, which could provide value for the early assessment of severity and the onset of treatment. However, ACE2 expression levels can be influenced by certain conditions, such as the following examples: 1. Lymphocytes and other immune factors are also negative modulators of serum ACE2 expression levels. Therefore, the decrease in serum ACE2 expression levels observed in COVID-19 patients, including those with high ACE2 expression, might be due to the downregulation of serum ACE2 by immune responses. 2. The viral load was not higher in the clusters with high serum ACE2 expression, contrasting with the viral concentration in feces and saliva, which might be caused by different sources of SARS-CoV-2 or higher detection limits in serum samples. Further studies will be necessary to provide more detailed explanations of these findings. (Alobaidy et al.2023)(Sabater et al.2022)(Pavel et al.2021) (Sabater et al.2022)(Mahmood et al., 2022)

Comparative analysis between mild, moderate, and severe patients. The viral infection and released inflammatory factors might regulate the genes expressed in serum. These expressed genes could be the markers for severity. However, compared with healthy people, the ACE2 levels of mild and moderate patients in each batch are significantly different, while that of severe patients is not. That is, compared with healthy people, only mild patients showed a significant difference in ACE2 gene expression levels, which may induce a different management strategy for severe patients.

Understanding gene expression requires close examination of the specific biology of the disease. In SARS-CoV infection in peripheral tissues including serum and lung samples, ACE2 is strongly down-regulated. SARS-CoV-2 and SARS-CoV are highly homologous enveloped viruses that cause respiratory disease in humans, so we cautiously suggest that ACE2 may, in turn, be used to evaluate the condition of COVID-19 patients. Though controversial, ACE2 plays an important role in mediating the interaction between the virus and target cells of the lower respiratory tract, thus increasing the pathogenicity of infection and over-activating the renin-angiotensin system.

ACE2, encoded on the X chromosome, is highly expressed in males, demonstrating a poor prognosis after SARS-CoV-2 infection. This may explain why more men than women develop the severe form of the disease. Our study discovered that ACE2 gene expression in the serum may be associated with the severity of COVID-19 patients, potentially used as a predictor of poor prognosis and a potential therapy approach for COVID-19 affecting the severity of patients. There are significant differences between the three stratifications of patients, suggesting its potential as a non-invasive severity

biomarker of COVID-19. Our study is based only on ACE expression results in the serum of patients with COVID-19, due to the restriction of virus interaction with ACE2. The previous ACE2 reports described that the receptor was identified in the lungs, esophagus, kidneys, bladder, and heart, which provide the possibility of its use in other tissue samples. However, conflicting results were found. So, whether or not ACE2 can be used as a non-invasive marker to evaluate the human 2019-nCoV strain infection requires further study and sample size widening. In summary, single-cell RNA sequencing can be used as a basis for studying changes in lung cells in COVID-19 patients.

More ACE-expressed cells were found and may be a severe factor for the COVID-19 virus. After collecting COVID-19 patients' serum samples, qRT-PCR was used to identify their gene expression profiles and hospitalized COVID-19 patient analysis in three different patients based on results. It was found that the gene between different stratifications is not independent but shows statistical significance. ACE2 gene expression may be associated with the severity of COVID-19 and potentially used as a non-invasive evaluation of a good prognosis. The epidemic and transmission trend of the imported positive cases in Luzhou, China, are difficult to fully understand because we performed single-stage reviewed research, analyzed fewer patient serum samples, and conducted limited cell function verification, and the relationship between ACE gene, ACE media, and 2019-nCoV infection was not fully investigated. (Al-Benna, 2020)(Gómez et al.2020)(Maza et al.2022)(Emilsson et al.2020)(Sabater et al.2022)(Martinez-Gomez et al.2022)(Daniel et al.2022)(Li et al., 2020)

5. Conclusion and Future Directions

In conclusion, ACE2 gene expression levels in the serum are closely related to disease severity in COVID-19 patients. In addition to the correlation between ACE2 expression and IL-6, the alternative splicing of the ACE2 gene had better diagnostic performance in severe cases. This does not directly account for a worsened prognosis, and the calling for intensive care support was only accepted in severe patient observation rather than a real biochemical signal indicating the need to go to the ICU. As we have only focused on a specific genetic background, further multicenter, large-scale studies should be carried out to verify the accessibility of our results to different populations. Longitudinal studies should also be performed to observe the dynamic gene expression changes of ACE2 in different stages of the disease treatment of COVID-19 patients. Furthermore, since host cell invasion by SARS-CoV-2 is based on entry via the human ACE2 serine protease complex, our results will provide possible therapeutic implications for a host-directed perspective in future research.

In view of the conclusions extracted from the results in the present study, our results showed that ACE2, a potential receptor for SARS-CoV-2, was highly expressed at the gene level in COVID-19 patients. The mRNA levels of ACE2 in the serum may give novel insights into the disease severity of COVID-19 patients. However, our study had several limitations. Firstly, our study was based on a single center, and the number of patients with severe and critical types was relatively small, which might lead to selection bias. Our results should be further verified in a multicenter study with a larger sample of COVID-19. Secondly, because the mRNA levels of ACE2 in the serum are not specific for COVID-19 in other coronavirus infections. In our future research, we will further develop a blind test for genes related to other environmental factors to improve specificity. Finally, potential physiological changes and complications of the patients were not addressed in combination with ACE2 expression. This not only enriches the concepts surrounding the disease but also paves the way for future research by other researchers.

5.1. Conclusion

This study finds a difference in ACE2 gene expression in the serum of different groups of COVID-19 patients, which is related to the severity of the disease. It is hoped that this will inspire both colleagues familiar with ACE2 and COVID-19 and infectious disease experts to further study these findings. As one of the susceptible factors of COVID-19 infection, ACE2 gene expression has not been systematically explored in the serum of patients with different severities of COVID-19. The huge sample size with high consistency and comparability obtained by high-throughput methods can more clearly reveal what we have been questioning: the association between ACE2 and the severity of COVID-19 patients.

This large sample size obtained by high-throughput methods ensures the availability and comparability of results.

ACE2 gene expression levels in the serum of COVID-19 patients were significantly correlated with the severity of the disease, especially in severe and non-severe as well as critical and non-critical COVID-19 patients. The severity of COVID-19 differs by age and gender. Compared with the younger group, ACE2 gene expression was significantly increased in the elderly group, and compared with male COVID-19 patients, the only significantly increased ACE2 gene expression was found in the serum of the female COVID-19 group. These findings are of some help for better understanding COVID-19 from a new point of view.

5.2. Recommendations for Future Research

Based on the findings of our study, we recommend that future research focus on several issues that are important for understanding the clinical significance of the gene expression levels of ACE2 in the serum of COVID-19 patients. First, further clarification is needed on the longitudinal impact of COVID-19 on ACE2 gene expression, particularly in larger and more diverse populations spanning different genders and age groups. This is also coupled with the investigation of possible drugs for COVID-19 treatment, as these drugs might influence the expression levels of ACE2 biomarkers, and either protect normal cells or contribute to the occurrence of associated symptoms. Second, other genes or non-protein-coding RNAs related to ACE2 expression should be further identified to determine their combined role in the body during SARS-CoV-2 infection. Additionally, defining the corresponding protein expression of ACE2 in the serum and tissues of COVID-19 patients is important for a comprehensive interpretation of the disease mechanism.

Third, considering our present research design, more comprehensive and representative follow-up studies with larger sample sizes are warranted in diverse populations to establish the association between ACE2 gene expression and SARS-CoV-2 infection and disease outcomes. This includes populations with different age ranges, various coexisting conditions, different genders, or those with more severe COVID-19 or admitted to the intensive care unit. Fourth, attention to technical issues related to the quantification of ACE2 protein in addition to the gene levels may facilitate the complete evaluation of the protein's potential role as a biomarker for COVID-19. The complexity of the structure and function of ACE2 may require collaborative research using multiple disciplines, such as infectious disease, immunology, genomics, proteomics, and metabolomics, to better understand the gene expression of ACE2 in the serum of COVID-19 patients. Therefore, to more thoroughly understand the clinical significance of ACE2, we recommend a detailed follow-up study to investigate the joint role of ACE2 with various clinical biomarkers in diagnosing and predicting COVID-19 disease progression.

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