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Study of Gene Expression of *ACE2* and *CXCL10* in COVID-19 Patients with and Without Chronic Diseases

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ABSTRACT:

Background :The coronavirus disease 2019 (COVID-19) is a potentially fatal respiratory disease caused by a new SARS-CoV-2, which was first identified in december 2019 in Wuhan (Hubei Province, China). Since then, it has rapidly spread worldwide, causing more than 150 million reported cases and over 3,1 million deaths globally since the start of the pandemic. COVID-19 has become the leading cause of morbidity and mortality in many countries and exemplifies the devastating impact of an emerging zoonotic pathogen on global public health and socio-economic development.

The aim of this study is determination of *ACE2* and *CXCL10* gene expression in patients of covid19 with and without chronic diseases and compare it with *ACE2* and *CXCL10* gene expression in control groups with and without chronic diseases, respectively, by using Real-Time PCR. The results showed that expression level of ACE2 in patients with chronic diseases higher than control group with difference significant (P=0.002). While, indicate that no there was difference significant in expression of *ACE2* gene in covid-19 patients of non-chronic diseases compare with control group (P=1.04). On the other hand, The results above has shown that levels of *CXCL10* gene expression is higher in COVID-19 patients, especially those with pulmonary problems, were significantly higher than those in healthy controls (P=0.0007). But, in patients of covid19 without chronic diseases our results found that non there was significant difference between levels of *CXCL10* gene expression COVID-19 patients compared with healthy controls.

Keywords: ACE2, CXCL10, Real time-PCR, Chronic diseases.

1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), is a highly contagious and pathogenic viral infection that caused a global pandemic with a substantial number of deaths (1). All coronaviruses have specific genes that code for proteins required for viral replication, nucleocapsid formation, and spike production(2). The classic method for coronavirus entry into host cells is receptor-mediated endocytosis(3). SARS-CoV-2 has been shown to enter the host cells through interactions with the angiotensin converting enzyme 2 (ACE2) receptor(4),with recognition of the ACE2 receptor by SARS-CoV-2 dependent on the structure of the coronavirus spike protein (5).

On December 31, 2019, the China Health Authority alerted the World Health Organization (WHO) to several cases of pneumonia of unknown etiology in Wuhan City in Hubei Province in central China. The cases had been reported since December 8, 2019, and many patients worked at or lived around the local Huanan Seafood Wholesale Market although other early cases had no exposure to this market (6). On January 7, a novel corona virus, originally abbreviated as 2019-nCoV by WHO, was identified from the throat swab sample of a patient (7). This pathogen was later renamed as severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) by the corona virus study group and the disease was named corona virus disease 2019 (Covid-19) by the WHO(8).

ACE2 is a protein-coding gene, is located along the short arm of the chromosome X (Xp22.2), the size of it is 39.98 kb, containing 20 introns, and 18 exons that can be transcribed into five different splice variants (ACE2-201 to ACE2-205); only two are protein-coding(Devaux *et al*., 2020). ACE2 is a metalloproteinase with a total length of 805 amino acids (9).

ACE2 gene is expressed in nearly all human organs in varying degrees. In the respiratory system, ACE2 is mainly expressed on type II alveolar epithelial cells, but weakly expressed on the surface of epithelial cells in the oral and nasal mucosa and nasopharynx, indicating that the lungs are the primary target of SARS-CoV-2. Moreover, ACE2 is highly expressed on myocardial cells, proximal tubule cells of the kidney, and bladder urothelial cells, and is abundantly expressed on the enterocytes of the small intestine, especially in the ileum(10). ACE2 is the cell receptor for SARS-CoV-2(11). Thus, the receptor-binding domain of the spike glycoprotein in SARS-CoV-2 binds to the tip of subdomain I of ACE2(12). Membrane fusion of the virus and the host cell is activated after binding, and viral RNA is subsequently released into the cytoplasm, establishing infection(9).

ACE2 is the angiotensin-converting enzyme 2 (ACE2), the homolog of ACE, is a catalytic component of renin-angiotensin system(RAS) (13). The RAS is a complex network that plays an important role in maintaining blood pressure as well as electrolyte and fluid homeostasis, affecting the function of many organs, such as the heart, blood vessels, and kidneys(9).

CXCL10 gene, localized on the chromosome 4 at band q21, contains 4 exons encoding a protein of 98 amino acids. CXCL10, previously referred to as interferon-γ (IFN-γ) inducible 10-kDa protein or IP-10, was isolated in 1985 by Luster (14). while treating a lymphoma cell line (U937) with recombinant IFN-γ, a cytokine with an important role in the induction and modulation of the immune responses.CXCL10 is chemokine has a molecular mass of 10 kDa and it is belong to the family of cytokines (15).

Moreover, it has been identified from several studies that chemokine CXCL10 is a very important factor that regulates many processes in the body, including immunity, angiogenesis and organ-specific metastasis of cancers. The role of CXCL10 in these processes makes it a promising therapeutic target for various diseases (16).

Also, it is modulating the course and the intensity of inflammation caused by SARS-CoV-2 (17). In response to SARS-CoV-2 and increased activity of IFN-γ, this chemokine is produced by a wide range of cell types including neutrophils, monocytes, endothelial, or dendritic cells(18). In turn, CXCL10 is selective ligand for CXCR3, which is mainly expressed on macrophages, T lymphocytes, dendritic cells, natural killer cells, and B cells. The CXCL10-CXCR3 axis has essential roles for the immune system. It has been revealed that normally it regulates immune cell differentiation, activation, and migration (19).

2. Material and Methods

Study Design

This study included Sixty sample of patients of COVID-19, 30 sample with chronic diseases(16 male and 14 female) and age range between(28-89)years, and 30 sample without chronic diseases(16 male and 14 female)with age range between(17-80)years. All samples of patients were collected from Al-hayat Center in Al-Zahraa Teaching Hospital, and from external laboratories after comfirming their infection using nose swab PCR test, in Wasit Province, Iraq.

While, samples of control groups involved 20 sample, 10 control samples with chronic diseases(4 male and 6 female) and age range between(30-60)years, and 10 control samples without chronic diseases(2 male and 8 female)with age range between(16-51)years. Control samples were collected from people not infected with covid-19 and was used covid-19 rapid test to prove it.

Methods

Samples of collection

Venous blood sample of 5 ml was drawn using a sterile syringes, which can be disposed of after use. Then, we put 5ml of blood into EDTA tube for RT PCR genetic testing, the sample was left for 15 minutes at a temperature of (20-25 °C) after which the sample was placed in the freezer at a freezing point -20 °C.

Quantitative Real-Time PCR (qPCR)

The quantitative Real-Time PCR used in quantification of ACE2 and CXCL10 genes expression analysis that normalized by housekeeping gene (GAPDH) in patient and healthy blood samples by using Real-Time PCR technique. Total RNA Extrac tion : Total RNA were extracted from blood samples by using (GENEzol™ TriRNA Pure Kit, cat# GZX100/D100) and done according to company instructions. cDNA synthesis includes DNase-I treated RNA samples were also used in cDNA using (Easy Script RT/RI Enzyme mix) and done according to company instructions.

The Real Time PCR primer that used in gene expression of ACE2 and CXCL10 gene and housekeeping GAPDH gene, they were designed in this study by using NCBI Genbank database and primers as following table (1).

ACE2	Forward	TCCATTGGTCTTCTGTCACCCG
	Reverse	AGACCATCCACCTCCACTTCTC
CXCL10	Forward	GGTGAGAAGAGATGTCTGAATCC
	Reverse	GTCCATCCTTGGAAGCACTGCA

Table (1): Primers Sequence Used in RT- qPCR

Sterile ddH2O was added as instructed by the manufacturer, using micopipett with barrier tips to resuspend the dried primer to a concentration of 100 µ M (the stock solution). To make a 10µM working solution of primer, 100µl stock was diluted 1:10 with sterile ddH2 as follows: To prepare 100 µl if working primer stock, 10µl of 100µM primer was added to 90µl of sterile ddH2O and stored -20°C

5-Preparation of qPCR master mix for (*ACE2,CXCL10*)

The Real-Time qPCR was used to quantification of the (ACE2and cxcl-10) expression via absolute method and analysis that was normalized by housekeeping gene (GAPDH) in the patients' group and control samples through utilized the technique of Real-Time qPCR that accomplished based on method manufacture company directive, ınserted at the following stages in table(2).

qPCR master mix	Volume
cDNA template (100 ng)	5 µL
Forward primer (10 pmol)	1 µL
Reverse primer (10 pmol)	1 µL
Free nuclease water	3 µL
qPCR Master Mix	10 µL
Total	20 µL

Table (2):The protocol of the qPCR master mix kit

The qPCR plate was ended loading, then the conditions of the thermocycler were applied as the following table(3).

Table (3): qPCR thermocycler stages.

qPCR step	Temperature	Time	Repeat cycle
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	20 sec	45
Annealing	60 °C	30 sec	
Extention Detection(scan)	75°C		

Statistical Analysis

The data were analyzed using the following software, Microsoft excel, IBM SPSSV26. The results reported in this study wereexpressed as mean± SD.

3. Results & Discussion:

In this study, molecular markers were studied for patients infected with Covid-19 with and without chronic diseases compared to control groups, using the Real-Time PCR (qPCR).

1. A: *ACE2* gene expression in Covid-19 patients with chronic diseases

The ACE2 gene that encode a protein called angiotensin-converting enzyme 2. This enzyme attaches to the outer surface of cells in the lungs, arteries, heart, kidney, and intestines. The ACE2 protein is part of the renin-angiotensin system (RAS), which regulates blood pressure and fluid and electrolyte balance, as well as systemic vascular resistance, which affects cardiac output (20).

This study shows that *ACE2* gene expressed in much higher level (P value =0.002) in covid-19 patients of chronic diseases in compare with corresponding level in control group of

chronic diseases. The mean was (2.1±0.26) for patients with chronic diseases, and (1±0.1) for control of with chronic diseases, shown in table (4), and figure(1).

Table(4):*ACE2* gene folding in covid-19 patients with chronic diseases and control groups

Group	Mean	±SD	SE	P-value
Patients of chronic disease	2.1	0.26	0.15	0.002
Control of chronic disease	1	0.1	0.06	

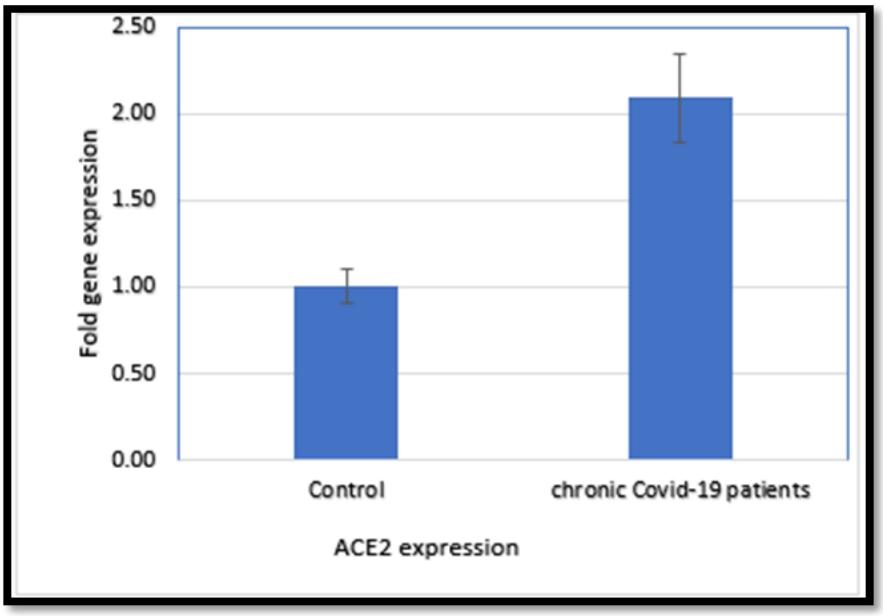

Figure (1): *ACE2* gene folding in covid-19 patients with chronic diseases and control group.

In results of our study indicate that expression level of ACE2 in patients with chronic diseases higher than control group with difference significant (P=0.002).

ACE2 is a counter-regulator of the renin angiotensin system (RAS). ACE2 cleaves angiotensin II (Ang II) into angiotensin 1 to 7 (Ang-(1–7)), leading to vasodilation (21). ACE2 provides protection against several chronic diseases, including cardiovascular diseases, lung injury, and diabetes (22).

In study by Pinto *et al*., (23), analyzed over 700 lung transcriptome samples from patients with comorbidities associated with severe COVID-19 and found that ACE2 was highly expressed in these patients compared to control individuals. This finding suggests that patients with such comorbidities may have higher chances of developing severe COVID-19. More particularly, in patients with chronic obstructive pulmonary disease (COPD), a lung RNA-seq dataset showed a significant upregulation in the expression of ACE2 in comparison to subjects with normal spirometry (controls) ($p = 0.00034$).

Patients with diabetes mellitus are at higher risk of COVID-19 and developing severe symptoms that are often fatal(24). ACE2 protein expression analyzed by immunohistochemistry in bronchial and alveolar samples displayed a significant increase in type 2 diabetic patients compared to the control group(25). Furthermore, microarray and RNA-sequencing expression data showed that the expression of ACE2 in pancreatic islets was significantly ($P < 0.05$) increased in diabetic patients compared to non-diabetic patients (26).

While, in Hypertension is one of the main comorbidities associated with worse outcomes in COVID-19. There was suggesting that hypertension may be associated with an up to 2.5-fold higher risk of severe and fatal COVID-19 (27). Thus, it would be relevant to explore the expression of ACE2 in hypertensive patients relative to non-hypertensive patients, but, there was scientific evidence concerning hypertension and COVID-19 focused almost exclusively on the possible effects of RAAS inhibitors(28).

1.B: *ACE2* gene expression in Covid-19 patients without chronic diseases

This study shows that no there was difference significant in expression of *ACE2* gene in covid-19 patients without chronic diseases compare with control group(P=1.04). The mean was (1.24±0.13) for patients without chronic diseases, and (1.00±0.07) for control of without chronic diseases, shown in table(5).

Table(5):*ACE2* gene folding in covid-19 patients without chronic diseases and control groups

Group	Mean	±SD	SE	P-value
Patients of non-chronic disease	1.24	0.13	0.07	1.04
Control of non-chronic disease	1.00	0.07	0.04	

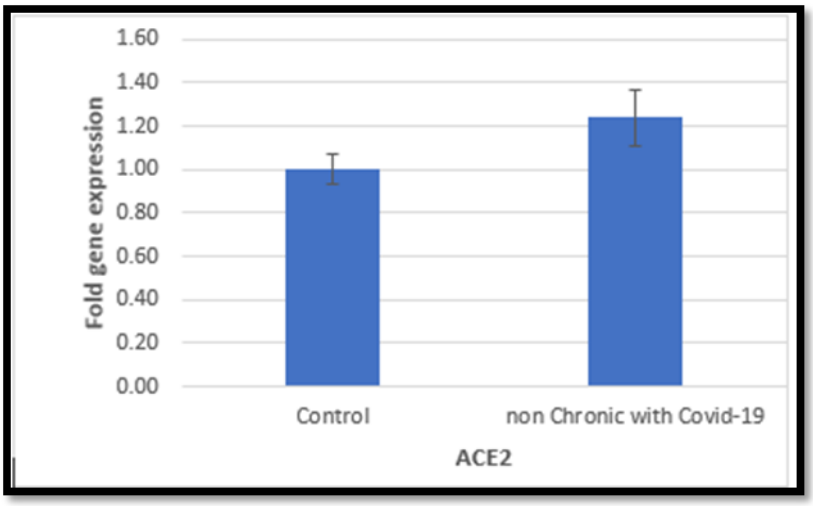

Figure (2): *ACE2* gene folding in covid-19 patients without chronic diseases and control group.

Table(5) above indicate that no there was difference significant in expression of *ACE2* gene in covid-19 patients of non-chronic diseases compare with control group(P=1.04).

In study by Kamel *et al*.,(29), include the mean ± SD values of angiotensin-converting enzyme-2 (ACE-2) activity levels was higher in the severe and moderate Covid-19 as compared to that found in healthy control (3.83 ± 0.82, 4.74 ± 0.85 and 3.03 ± 0.82 ng/ml) respectively and it's reveal a non-significant differences between moderate and control groups (P = 0.721) and significant differences between sever Covid-19 and apparently health control group (P < 0.001).

While, in study by Alobaidy *et al*., (30), found that significantly lower expression levels of ACE2 in COVID-19 patients compared to a hypothesized value (1.0) for controls. Median ACE2 fold change(FC) (FC = 0.05, 95% CI for the median = 0.0.017 to 0.098). This was statistically significantly lower than the normal control value of 1.0 (P < 0.001). This due to SARS-CoV-2 may induce downregulation of ACE2 by a number of mechanisms including: decreased ACE2 receptor expression due to immune dysfunction; enhanced shedding of

membrane-bound ACE2; and endocytosis of ACE2 receptor with SARS-CoV-2 (31). Downregulation of ACE2 has been shown to alter the ratio of ACE to ACE2 in many pathological conditions(32). Accordingly, high ACE levels may suggest low ACE2 levels and vice (31).

2.A: Gene expression of *CXCL10* in covid19 patients with chronic diseases

This research has shown that levels of CXCL10 gene expression is higher in COVID-19 patients, especially those with pulmonary problems, were significantly higher than those in healthy controls(P=0.0007). The mean was (5.79) for patients of chronic diseases, and (1.10)for control group of chronic diseases, shown in table() and figure().

Table(6): *CXCL-10* gene folding in covid-19 patients whom had a chronic diseases and control group.

Group	Mean	±SD	SE
Patients of chronic disease	5.79	0.68	0.39
Control of chronic disease	1.10	0.51	0.30
P-value	0.0007		

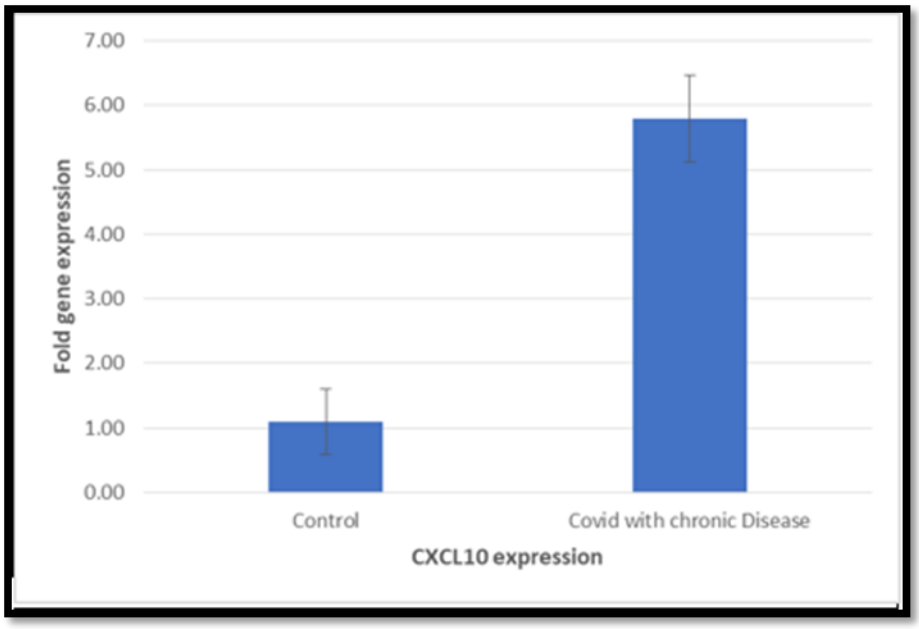

Figure (3): *CXCL-10* gene folding in covid-19 patients whom had a chronic diseases and control group

The current study, found that levels of CXCL10 gene expression is higher in COVID-19 patients, especially those with pulmonary problems, were significantly higher than those in healthy controls(P=0.0007).

CXCL10 is a pro-inflammatory chemokine, CXCL10 may be one of immune-related genes in the body after COVID-19 infection, and tried to identify the key chemokine CXCL10 that causes the cytokine storm, and it is expected to become the therapeutic target(33).

CXCL10 is elevated in several diseases, including hepatitis B, tuberculosis, cancer, diabetes, and autoimmune disorders(34). CXCL10 is the most widely studied chemokine in patients with COVID-19. It has consistently been found to play a principal role in the cytokine storm induced by COVID-19(35). High CXCL10 levels are strongly associated with the infiltration

of immune cells into the alveolar space, peribronchial, and perivascular regions, and pulmonary damage(36). Significantly higher serum concentrations of CXCL10 and viral loads in patients with COVID-19 have been reported in patients with fatal disease than in survivors, and a CXCL10 levels are positively correlated with the SARS-CoV-2 viral load(37).

CXCL10 levels differ also according to the severity of the disease and they is the highest in critically ill patients with COVID-19. This indicated that a high level of CXCL10 as a result of SARS-CoV-2 infection should be considered as having a potentially high risk of complications or severe course of COVID-19. In addition, knowing that infection of SARS-CoV-2 induces "cytokine storm"(38).

2.B: Gene expression of *CXCL10* in covid19 patients without chronic diseases

In patients of covid19 without chronic diseases, our results found that non there was significant difference between levels of CXCL10 gene expression COVID-19 patients compared with healthy controls. The mean was (1.30) for patients of non-chronic diseases, and (1.00) for control group of non-chronic diseases, shown in table, shown in figure (4).

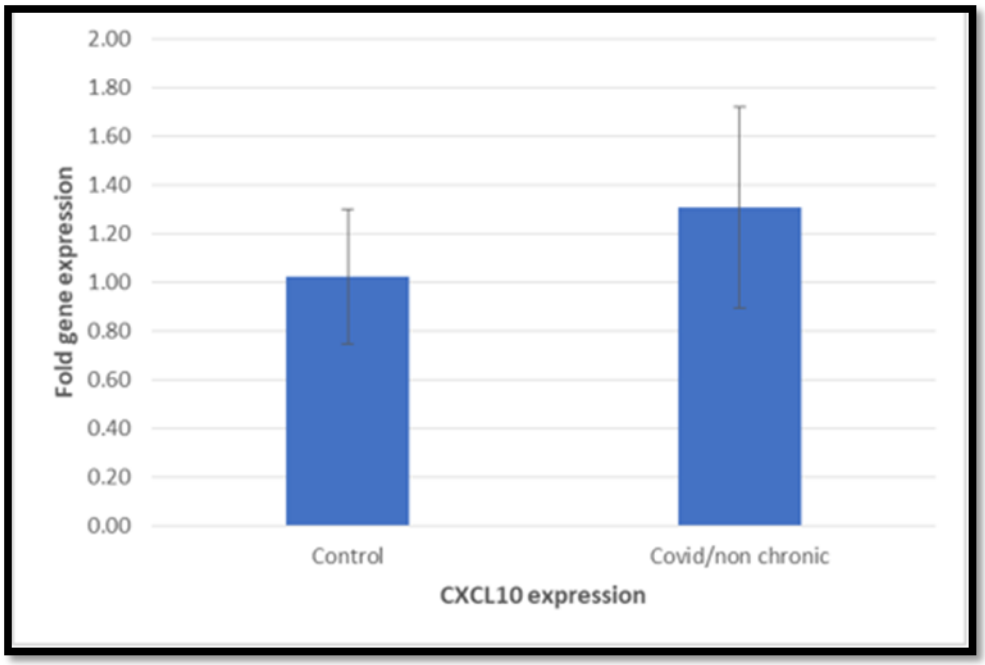

Figure (4): *CXCL-10* gene folding in covid-19 patients of non- chronic diseases and control group

The results of our study showed that non there was significant difference between levels of CXCL10 gene expression COVID-19 patients compared with healthy controls.

In study by Çelik *et al*., (39), showed that CXCL10 level was significantly higher in patients with severe COVID-19 group than in those with moderate disease. CXCL10 level was significantly higher in both patient groups than in the control group($P<0.001$)**.** Also, in study by Hameed *et al*., (40), found that CXCL10 gene expression upregulated to 35 fold in SARS-C0V-2 infected compared to the uninfected people.

4. Conclusion:

- Increased expression of *ACE2* gene in Covid19 patients of chronic disease compared with control group due to ACE2 provides protection against several chronic diseases, including cardiovascular diseases, lung injury, and diabetes. But, decrease expression of *ACE2*

because SARS-CoV-2 may induce immune dysfunction; enhanced shedding of membrane-bound ACE2; and endocytosis of ACE2 receptor with SARS-CoV-2.

- Increase expression of *CXCL10* in patients of covid19 with chronic disease compared with control group due to CXCL10 is a pro-inflammatory chemokine, it may be one of immune-related genes in the body after COVID-19 infection, and tried to identify the key chemokine CXCL10 that causes the cytokine storm, and it is expected to become the therapeutic target.

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