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Correlation of Expression of MMP-2, ACE2, and TMPRSS2 Genes with Lymphopenia for Mild and Severity of COVID-19

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ABSTRACT

Some risk causes may be associated with the severity of COVID-19. The central host-pathogen factors might affect infection are human receptor angiotensin-converting enzyme 2 (ACE2), trans-membrane protease serine 2 (TMPRSS2), and SARS-CoV-2 surface spike (S)-protein. The main purpose of this study was to determine the differences in the expression the metalloproteinases-2 (MMP-2), MMP-9, ACE2, and TMPRSS2 genes and their correlation with lymphopenia in the mild and severe types of the COVID-19 patients.

Eighty-eight patients, aged 36 to 60 years old with the mild (n=44) and severe (n=44) types of COVID-19 were enrolled. Total RNA was isolated from the peripheral blood mononuclear cells (PBMCs). The changes of MMP-2, MMP-9, ACE2 and TMPRSS2 gene expression in PBMCs from mild and severe COVID-19 patients were examined by the real time-quantitative polymerase chain reaction (RT-qPCR) assay and, compared between the groups. Data were collected from May 2021 to March 2022.

The mean age of the patients in both groups was 48 (interquartile range, 36–60), and there were no appreciable differences in age or gender distribution between the two groups. The present study showed that a significant increase in the expression of ACE2, TMPRSS2, MMP-2, and MMP-9 genes in the severe type of the COVID-19 patients compared, to the mild type of the COVID-19 patients.

Overall, it suggests the expression levels of these genes on the PBMC surface in the immune system are susceptible to infection by SARS-COV-2 and therefore could potentially predict the patients' outcome.

Keywords: ACE2; COVID; Gene; Lymphopenia; Matrix metalloproteinase

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INTRODUCTION

The coronaviruses diseases-2019 **(**COVID-19) pandemic is caused by the novel coronavirus, which the International Committee on Taxonomy of Viruses (ICTV) eventually designated as the severe acute respiratory syndrome coronavirus-2 (SARS-COV-2). SARS-COV-2 was first discovered in the Chinese province of Wuhan and spread mostly through human-to-human contact while people were migrating.[1-4] Although the virus's origin, natural reservoir, and intermediate host are still up for debate, it has spread over the world and wreaked devastation on the human race. While type-2 diabetes (T2D), cardiovascular diseases (CVD), hypertension (HT), and compromised immunity (CI) are recognized as key comorbidities of COVID-19, the death rate among older people (age > 60 years) is shown to be much greater.[5-7]

The three main host-pathogen determinants known to affect infection are human receptor angiotensin-converting enzyme 2 (ACE2), trans-membrane protease serine 2 (TMPRSS2), and SARS-COV-2 surface spike (S)-protein.[8] Other membrane proteases that are known to be critical in the Middle East respiratory syndrome coronavirus (MERS-COV) and SARS-COV infections, including CD26/DPP4, trypsin, cathepsin L, and TMPRSS11d, were also believed to be crucial in the development of COVID-19.[9] Recent investigations have emphasized the significant importance of ACE2 and TMPRSS2 in the pathogenesis of SARS-COV-2 infection.[10,11] To get ready and enter the human cell, viral S-protein physically interacts with and makes use of TMPRSS2 and ACE2 which are linked to membranes. The affinity of SARS-CoV-2 RBD to ACE2 has been dramatically boosted by a factor of 10 to 15 when compared to SARS-COV due to amino acid changes at critical locations on RBD.[3,12,13]

ACE2 gene has 40 kbp, which is located at position Xp22.2 and has 18 exons. This gene encodes a membrane glycoprotein with a weight of 100 KD and 805 amino acids, which is one of the zinc-containing metalloproteinases ACE2.[12] ACE2 Acts as a carboxymanopeptidase that can hydrolyze angiotensin II, our knowledge about ACE2 dates back to its discovery (20 years ago).[14] ACE2 expression level is different in different tissues.[15] The TMPRSS2 gene is located at 21q22.3 and produces a membrane serine protease that is regulated by androgens.[16] TMPRSS2 expression has been reported on the surface of epithelial cells of the upper and lower respiratory system. Spike protein of SARS-COV-2 binds to the ACE2 receptor, and this virus uses TMPRSS2 to cleave the S protein at the S2/S1 site, allowing the integration of the virus and the cell membrane.[17] Because lymphocytes contain the angiotensin-converting enzyme 2 (ACE2), the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) may also target them, activating them and causing activation-induced cell death that may result in both CD4+ and CD8+ T cell lymphopenia.[18] Since SARS-COV uses ACE2 and TMPRSS2 to attack human cells, identifying their expression levels at the cell level is the key to understanding the pathogenesis of SARS-COV-2.[13,19]

In the lung, SARS-COV-2 mainly infects monocytes and macrophages,[20,21] but the expression of ACE2 is not limited to the lungs, and extrapulmonary spread of SARS-COV-2 has been observed. In lung illness, the metalloproteinases (MMPs) play crucial functions.[19,20] Metalloproteinases-2 (MMP-2) and MMP-9 have both been proposed as possible biomarkers for septic patients due to the severe form of COVID-19's many similarities to sepsis.[22-24] Matrix metalloproteinases (MMPs) are neutral proteinases that play a role in the degradation and regeneration of ECM under various physiological and pathological conditions and cause the degradation of ECM proteins such as type IV collagen, elastin, vitronectin, and aggrecan.[4] An increase in the number of matrix metalloproteinases MMP-2 and MMP-9 in the plasma of patients with COVID-19 is associated with a high risk of mortality.[25] Also, increased MMP-2 levels in patients with COVID-19 may reflect an overactive renin-angiotensin system (RAS),[26,27] and high levels of angiotensin (Ang) II observed in COVID-19 disease are associated with endothelial damage.[28] Since SARS-COV-2 attacks all human cells, identifying their expression levels at the cell level is the key to understanding the pathogenesis of SARS-COV2.[13] In the present study to evaluate correlation of lymphopenia in lymphocyte subsets with different gene expression Human peripheral blood mononuclear cells (PBMCs) were used. Therefore, the aim of present study was to investigate the differences in the expression MMP-2, MMP-9, ACE2 and TMPRSS2 genes and their correlation with lymphopenia in the mild and severe types of the COVID-19 patients.

MATERIALS AND METHODS

Study Design and Patients

In the present study, a total of 88 individuals, comprising 44 severe COVID-19 instances and 44 mild COVID-19 cases, who diagnosed with mild and severe types of COVID-19 were included. The patients had symptoms of COVID-19 were from those who referred to Khorshid Hospital in the Isfahan, Iran. Sampling of patients were collected from May 2021 to March 2022.

In patients with severe COVID-19, there were 23 (52.3%) male cases and 21 (47.7%) female cases, and in patients with mild COVID-19, there were equal numbers of male and female patients. The mean age of the patients in both groups was 48 (interquartile range, 36–60), and there were no appreciable differences in age or gender distribution between the two groups.

The inclusion criteria for the mild type of disease were defined as those patients, who were diagnosed with the COVID-19 polymerase chain reaction (PCR) test and had PO2>94%.[29,30] The inclusion criteria for the severe type of disease were the positive PCR test together with the multiple symptoms including PO2<94%, PaO2/FiO2<300 mm Hg, respiratory rate>30 breaths/min, and lung infiltrates>50%.[29,30] However, the exclusion criteria for the mild and sever types of COVID-19 were history of vaccinated COVID-19**,** the diabetes mellitus, heart disease, dyspnea, immune-deficiency and/or autoimmune disease, shortness of breath, abnormal chest imaging and hypertension. Informed consent was obtained from all persons before participating in this study, which was approved by the Ethical committees of Isfahan university medical of science, Isfahan, Iran, (IR.ARI.MUI.REC.1400.061).

Sample Size

The optimum sample size predictable by the formula the survival analysis formula:

$$n = \frac{(z_1 + z_2)^2 (2S)^2}{d^2}$$

However, with the hypothesis of a 95% confidence level and a study power of 80%, equal number of cases. We calculated the number of patients where, z_1 = 1.96, Z_2=0.84, S=It is an estimate of the average standard deviation of each variable in two groups, d=The minimum difference in the average of each variable between the two groups shows that the difference is significant, and S is considered to be 0.6. According to the calculation, the number of sample size was 44 (44 controls, which were patients with mild type of COVID-19 disease and 44 people with severe type of COVID-19 disease). However, we included 88 COVID-19 patients from May 2021 to March 2022.

Isolation of PBMCs

According to the manufacturer's recommendations, peripheral blood mononuclear cells (PBMCs) were separated using a Ficoll-Hypaque gradient (Lymphodex, Inno-Train, Germany). Blood samples were drawn, and collected in EDTA-containing tubes, and then mixed with an equivalent volume of Ca2+- and Mg2+-depleted phosphate-buffered saline (PBS, pH 7.3) in 15 ml conical tubes. The mixture was carefully deposited on top of a Ficoll- -Hypaque solution with a sample-to-Ficoll ratio of 2:1. After 25 minutes of centrifugation (at 2800 rpm) the middlephase (white layer) was extracted as PBMCs. The collected cells were washed in PBS (2x) before being counted and tested for viability using a hemocytometer and trypan blue. For the following investigations, cells with a minimum of 75% viability were used.

RNA Extraction and Real-time PCR Experiments

Using an RNA Extraction Kit (Co.Pars Tous, Iran), total RNA was extracted from PBMCs as directed by the manufacturer. After that, using a nanodrop 2000 to measure absorbance at 260 nm and 280 nm, the quantity, and quality of extracted RNA were measured (NanodropTM 2000; Thermo Fisher Scientific, USA). Analysis of the expression of MMP-2, MMP-9, ACE2, and TMPRSS2 at 260 and 280 nm wavelengths, total RNA extracted from PBMCs of severe and mild COVID-19 patients was quantified for concentration and purity. The quality of the extracted RNAs was further assessed using agarose gel electrophoresis (Figure 1).

Then, using the Bio fact 2X RT PCR Master Mix kit (Bio fact, Korea) and oligo-dT primers, 5 µg of the total RNA was used to create cDNA. The AlleleID 7.6 program from Premier Biosoft was used to create the PCR primers for this investigation, and Metabion GmbH (Planegg-Martinsried, Germany) manufactured them (Supplementary Table). The StepOne PlusTM Real-time PCR detection equipment and 2X SYBR Green Real-Time PCR High ROX (Pars Tous, Iran) were used to

execute real-time polymerase chain reactions in a total volume of 20 µL (Applied Biosystems). The PCR amplification settings included a denaturation stage at 95°C for 15 mins, followed by 45 cycles of annealing at 60°C for 30 s, and extension at 72°C for 30s. The melting temperature of specific amplification products and primers was found via melting curve analysis. At least three times each, these experiments were independently replicated in duplicate. As an endogenous control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used. According to a prior description, the expression level of each target gene was computed as $2^{-\Delta\Delta Ct}$ (Supplementry Table).

Statistical Analysis.

Using SPSS, all of the statistical analysis was done (IBM Statistics Software V.25). The GraphPad Prism version 5.0 was used to illustrate the figures and the results were expressed as the mean±SD. The normality of data was assessed using the Kolmogorov-Smirnov test. The statistical comparisons using the Mann-Whitney test which it was used to compare the data sets. This study used the Bivariate, Spearman for correlation. A *p*-value of 0.05 or less was deemed statistically significant.

RESULTS

MMP-2, MMP-9, ACE2, and TMPRSS2 Gene Expression

The comparative expression of MMP-2, MMP-9, ACE2, and TMPRSS2 genes expression in PBMCs, mild and severe types of COVID-19 cases was Significant ($p<0.05$). From mild and severe types of COVID-19 patients, the fold changes of MMP-2(6.2±2.8, 7.3±1.4), MMP-9 (5.7±3, 7.4±1.2), ACE2 (5.1±2, 13.9±32) and TMPRSS2 (5.2±2, 14.4±2.7) genes in PBMCs were expressed as Mean±SD, individually (Figure 1, Table 1).

The Correlation of Lymphopenia with the Expression Levels of MMP-2, MMP-9, ACE2, and TMPRSS2

The results showed significant linear correlation between the lymphopenia with the expression of MMP-2, MMP-9, ACE2, and TMPRSS2 genes in the PBMCs of the mild and severe COVID-19 patients ($p<0.05$, Table 2).

The TMPRSS2 gene exhibited the most significant correlation with the status of lymphopenia among either the mild and severe types of COVID-19 patients in the Bivariate correlation analysis. The R values also showed a reverse interaction between the transcript levels of the investigated genes as well as IL-6 with the lymphocyte counts in the mild and severe types.

Table 1. Comparative analysis of MMP-2, MMP-9, ACE2, and TMPRSS2 gene expression in PBMCs, among mild and severe COVID-19 patients.

Gene	Group	Cases (n)	Mean (±SD)	*p*
ACE2	S	44	13.893 (2.293)	<0.001
	M	44	5.100 (2.237)	
TMPRSS2	S	44	14.394 (2.792)	<0.001
	M	44	5.281 (2.479)	
MMP-2	S	44	7.308 (1.436)	<0.028
	M	44	6.262 (2.742)	
MMP-9	S	44	7.490 (1.240)	<0.01
	M	44	5.757 (3.008)	

S; Severe, M; Mild, ACE; Angiotensin-converting enzyme, TMPRSS; Trans-membrane protease serine, MMP; Metalloproteinasesh

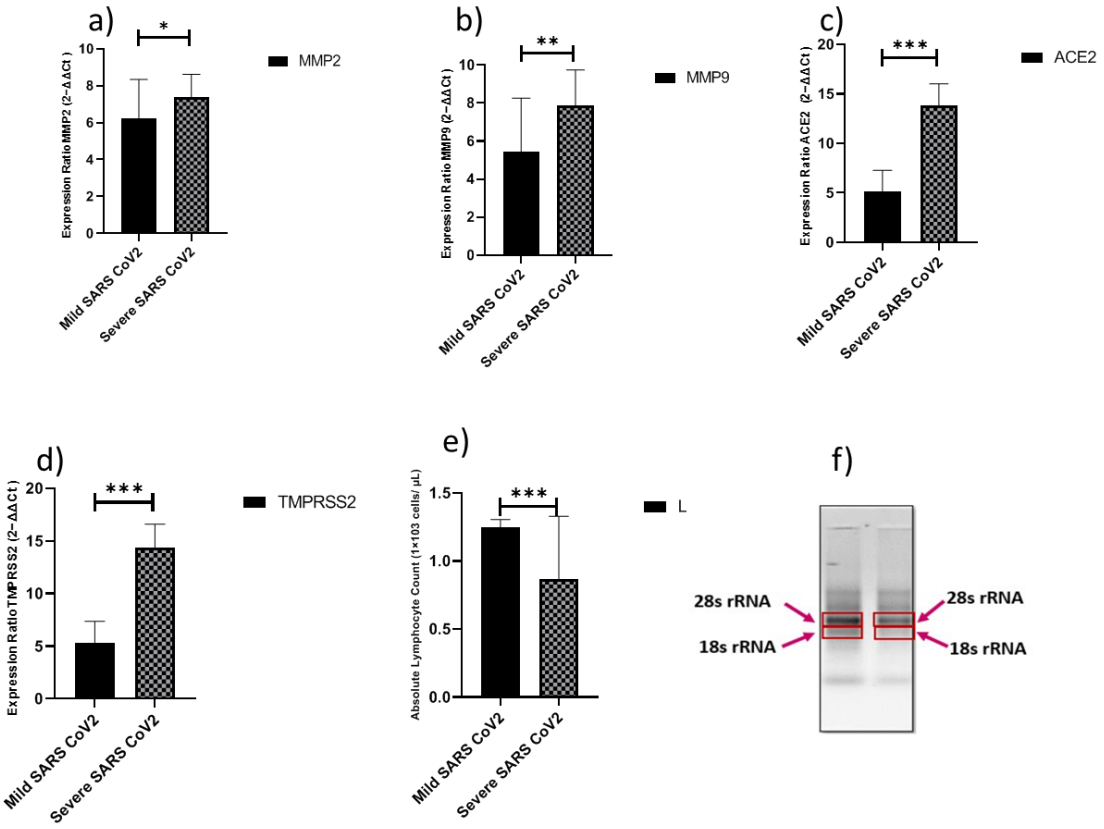

Figure 1. The relative expression of MMP-2, MMP-9, ACE2, and TMPRSS2 genes expression in PBMCs, mild and severe types of COVID-19 cases. The fold changes of a) MMP-2, b) MMP-9, c) ACE2 and d) TMPRSS2 genes in PBMCs from mild and severe types of COVID-19 patients were examined by at least three different real-time PCR experiments and were expressed as Mean ± SD. e) The differences in the absolute counts of lymphocytes in two groups of COVID-19 patients (mild vs severe) were expressed as bar charts, f) Agarose gel electrophoresis of blood total RNA. * p<0.05, ** p<0.01 and *** p<0.001.

Table 2. The relationship between MMP-2, MMP-9, ACE2, and TMPRSS2 gene expression, with lymphopenia among mild and severe COVID-19 patients.

			Variables							
Groups	**Patients (no.)**		**ACE2**		**TMPRSS2**		**MMP2**		**MMP9**	
Mild	44	Lymphocyte	R	P	R	P	R	P	R	P
			-0.240	0.021	-0.360	0.01	-0.340	0.025	0.345	0.001
Severe	44	lymphocyte	R	P	R	P	R	P	R	P
			-0.552	0.001	-0.740	0.002	-0.644	0.013	-0.206	0.021

S; Severe, M; Mild, ACE; Angiotensin-converting enzyme,TMPRSS; Trans-membrane protease serine
MMP; Metalloproteinasesh

DISCUSSION

SARS-CoV-2 infection by respiratory droplets and close contact is thought to be the primary cause of COVID-19 person-to-person transmission.[31,32] The spike glycoprotein of the COVID-19 and the host cellular SARS-COV receptor ACE2 facilitate SARS-COV-2 infection and cellular entrance.[6,9] By cleaving ACE2 to facilitate viral uptake and activating the SARS-COV-2 spike protein for tissue fusion, TMPRSS2 is necessary to promote SARS-CoV entry besides ACE2.[8,11,33] But despite the increase in research on SARS-COV-2 infection, whether many cells can be infected by this virus or not still is unknown.[34,35] The key purpose of this study was to determine the variability in the expression of MMP-2, MMP-9, ACE2, and TMPRSS2 genes and their association with lymphopenia in the COVID-19 patients.

The results of our study on the level of gene expression ACE2 and TMPRSS2 showed an increase in the PBMCs. These results were similar to others studies,[36,37] which suggested a possible pathophysiological mechanism for the involvement of the central nervous system (s) (CNS), where the virus could be able to cross the blood-brain barrier via hematological diffusion or via bind to the epithelial ACE2 receptor. Another study confirms our result indicating that SARS-COV-2 mainly infects monocytes and macrophages, but the expression of ACE2 is not limited to the lungs, and extra-pulmonary spread of SARS-COV-2 has been seen.[8] Virus invasion of monocytes, macrophages and excessive production of cytokines (cytokine stromal) which is related to the severity and prognosis of the infection.[38] Other research has shown that cells including monocytes, dendritic cells, and T lymphocytes in the PBMC of COVID-19 diseases express ACE2 and TMPRSS2, which can increase the progression of the disease.[35] Likewise, another study has revealed that about 8-40% of PBMCs expressed ACE2 on their surface.[26] Li and his colleagues investigated the expression of the ACE2 gene as the cellular receptor of SARS-COV-2 in different human tissues. Therefore, it seems immune responses to viral infection in different hosts are different, and it is because of the difference in the relationship between ACE2 and the immune indicators of different tissues.[15]

As earlier explained, MMPs are neutral proteinases that play a role in ECM degradation and transformation in many physiological and pathological disorders.[4,39] MMP-2, is one of these enzymes that are especially main in the pathogenesis of infectious, inflammatory and neoplastic diseases in various organs such as lung. Concerning the role of this enzyme, it could cause the break of ECM proteins such as collagen (type IV), elastin and etc.[4] The results of the present study on the level of gene expression of MMP-2 and MMP-9 showed an increase in the PBMCs. Similarly, it has been shown that the increase in the level of MMP-2 protein in the plasma of COVID-19 patients indicates severe inflammation like that in septic disease.[24] Therefore, it suggests increased MMP-2 levels in patients with COVID-19 may reflect an overactive rennin-angiotensin system (RAS).[26,27] In Parallel with our results, levels of angiotensin (Ang) II could be associated with endothelial destruction[28] and increase the expression of MMP-2 protein in plasma and causes a severe type of coronavirus.[40] In addition, Karolina et al, revealed that the rise in the MMP-2 and MMP-9 is associated with a great threat of death in the patients with COVID-19.[25,41]

However, the restriction of the present study was the low number of under-investigated patients and it would be improved generalized. It is more expansive examined in the forthcoming, and it needs more research at protein levels between severe and mild patients compared to healthy controls.

Overall, the correlation analysis of the mRNA levels of the MMP-2, ACE2, and TMPRSS2 genes with lymphopenia for mild and severity of COVID-19 determine that the entry of the virus as the initial stage in viral pathogenesis is well-thought-out and also noteworthy association between these genes and the lymphocyte counts which it could be applied for the purpose prognosis of COVID-19 disease.

STATEMENT OF ETHICS

The study was approved by the Ethical committees of Isfahan university medical of science, Isfahan, Iran, (IR.ARI.MUI.REC.1400.061).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest concerning this study.

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