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Severe Acute Respiratory Syndrome Coronavirus 2 Gene Expression as a Prognosis Predictor for COVID-19

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Abstract

INTRODUCTION: Real-time quantitative PCR (RT-qPCR) is the gold standard for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is specific, sensitive, and simple quantitative. The target of RT-qPCR is to assess the expression level of the SARS-CoV-2 gene through cycle threshold values (CT-value).

AIM: The purpose of this study was to analyze the association between the level of SARS-CoV-2 gene expression and the severity of COVID-19 in patients hospitalized.

METHOD: This research is an analytic observational study with the cross-sectional method. While the research sample was taken using a consecutive sampling technique from the Medical Records of Sumedang Hospital and Cideres Hospital, West Java, Indonesia, from December 2020 to March 2021. Patient parameters include analysis of age, sex, comorbidity, and disease severity. The severity of the patient is classified based on complaints and oxygen saturation. The expression level of the SARS-CoV-2 N gene and E gene was assessed by calculating the relative quantification by comparing the expression of the E and N gene with the expression of the internal control gene by the Livak formula ($2^{-\Delta\Delta CT}$ Formula).

RESULT: The Spearman correlation test showed that there was a relationship between the expression of SARS-CoV-2 genes E and N genes with the severity of COVID-19 patients (with $r = 0.374$ and $p < 0.0001$) and (with $r = 0.452$ and $p < 0.0001$).

CONCLUSIONS: There is a correlation between the level of expression of genes E and gene N with the severity of patients.

Introduction

On March 11, 2020, the World Health Organization (WHO) declared the disease outbreak due to the COVID-19 coronavirus is a global pandemic. The COVID-19 pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. COVID-19 has infected over 200 million people worldwide, and more than 4 million people have died from it [2].

Patients COVID-19 have a range of symptoms and clinical manifestations, such as disturbances of smell and taste, sneezing, fever, cough, sore throat, wheezing, diarrhea, nausea and vomiting, shortness of breath, respiratory failure, and up to dyspnea with hypoxia associated with lung infiltration [3]. The patient's condition may suddenly worsen. The pathogenicity of COVID-19 begins with the entry of the virus into the human body. It replicates in the body by forming a bond between the viral protein and the angiotensin-converting enzyme 2 (ACE2) receptor found in the ciliary cells of the nasal epithelium, respiratory tract, and type II alveolar cells. This binding will mediate the entry of the virus into cells which will cause damage to the endothelium and epithelial structures. It results in increased permeability and accumulation of protein-rich fluid in the interstitial and alveolar spaces, resulting in severe pneumonia, RNAemia, combined with ground-glass opacity, and acute myocardial infarct [4], [5], [6].

Diagnosis of COVID-19 is carried out by detecting the SARS-CoV-2 virus through several methods such as antibody tests, SARS-CoV-2 specific antigens, and other serological tests such as rapid diagnostic tests [7], [8]. Real-time quantitative PCR (RT-qPCR) is the gold standard for detecting SARS-CoV-2 because it

is specific, sensitive, and semi-quantitative. The target of RT-qPCR is to assess the expression level of the SARS-CoV-2 gene through the cycle threshold value (CT-value). The target genes for RT-qPCR are structural genes in SARS-CoV-2, including glycoprotein spike (S), envelope (E), transmembrane (M), helicase (Hel), nucleocapsid (N), and RNA-dependent RNA polymerase (RdRp). The sample used in this examination is the oropharyngeal or nasopharyngeal swab [9].

The expression level of the SARS-CoV-2 structural gene can predict the viral load found in COVID-19 patients, this data can be obtained by comparing the CT internal control as a housekeeping gene with the CT gene SARS-CoV-2. One of the genes often used as a target for RT-PCR is gene E and gene N, which are envelope and nucleus genes (nucleocapsid). A high viral load can affect the clinical manifestations and mortality of COVID-19 patients, which is the standard for diagnosis of COVID-19 [10], [11]. The higher the expression level, the higher the viral load or RNAemia, and should be a predictor of the course of the disease and prognosis. However, at this time, the PCR assay is carried out imprecisely, or only to confirm the presence or absence of a virus. Therefore, determining markers as predictors of prognosis are urgently needed to prevent worsening or even death [12], [13], [14]. The purpose of this study was to analyze the relationship between the level of SARS-CoV-2 gene expression and the severity of COVID-19 patients hospitalized at the Regional General Hospital in West Java.

Method

This study used an analytic observational research design with the cross-sectional method. Sampling was conducted using a consecutive sampling technique from medical records at Sumedang and Cideres regional general hospitals, West Java, Indonesia, from December 2020 to March 2021. Inclusion criteria included complete medical record data from patients undergoing COVID-19 in inpatient installations. In contrast, the exclusion criteria are CT of Internal control SARS-CoV-2 from RT-PCR unidentified. Patient parameters include analysis of age, sex, comorbidity, and disease severity. In addition, a Real-Time PCR assay was done when the patient was admitted to the Emergency Unit. Patients are tested positive for COVID-19 if a CT value of one or more SARS-CoV-2 genes targets RT-PCR, such as nucleocapsid (N) and envelope (E). Total RNA was extracted from nasopharyngeal and oropharyngeal swab samples using the Promotor Nucleic acid extraction kit (REF P121-1301-ACON Biotech, Hangzhou). N and E genes' expression was measured with the qRT-PCR method quantified by an Applied Biosystems 7500 real-time PCR using Promotor SARS-CoV-2 RT-PCR test kit (REF P131-1581- ACON Biotech, Hangzhou), according to manufacturer's protocol.

The relative expression level was calculated using relative quantification by comparing the expression of the E and N genes with the internal control as a housekeeping gene calculation using the Livak formula ($2^{-\Delta\Delta CT}$) [15]. The controls used in this study were ten asymptomatic patients who were confirmed positive by real-time PCR examination using the same tools and reagents.

Ethical clearance

The use of subjects and clinical data from patients in this study has received approval from the Council of the Ethics Committee for Medical and Health Research Universitas Islam Bandung, Indonesia, on May 31, 2021 (Reference: 103/KEPK-UNISBA/VII/2021), which has met the rules under the Declaration of Helsinki. The confidentiality of patient data is maintained by keeping patient personal data confidential.

Diseases severity

The patient's parameters include analysis of age, sex, fatality rate, bronchopneumonia, and disease severity. Classification of case severity in COVID-19 patients was divided into mild, moderate, severe, and critical. Patients are categorized as mild severity if they experience symptoms of fever, myalgia, cough, without shortness of breath, and oxygen saturation above 93%. Patients are classified as moderate severity if they experience typical symptoms of pneumonia such as fever, cough with phlegm, and dyspnea, with definitive lung lesions confirmed by chest X-ray. Severity defines if there are one or more of the following criteria present: respiratory rate of 30 bpm or more, oxygen saturation (SpO_2) <93% at rest or arterial oxygen pressure to the fractional inspired oxygen concentration of 300 mm Hg or less, or lesions present more than 50% of the lung as evidenced by Chest X-ray during the last 48 h. Finally, patients were considered critical if they met one or more of the following: Requiring intubation because of respiratory failure, shock, and multiple organ dysfunction requiring intensive unit care [16].

Statistical data analysis

The correlation between gene expression level and severity using the Spearman correlation test; a $p < 0.05$ was considered significant. The data were tabulated and then processed using Statistical Package for GraphPad Prism Version 9.2.0 (322).

Results

Population characteristic

The total number of patients from two hospitals was 390, matching the inclusion criteria while excluding 286 patients. More than 50% of patients are female, and the most age range is 41–60 years (54.19%). Patients with comorbidities were 175 patients (61.2%), patients with diabetes mellitus were 99 patients (34.6%), hypertension 92 patients (32.1%), COPD/asthma two patients (0.7%), cardiac in 20 patients (7%), malignancy in one patient (0.3%), and other comorbidities in 109 patients (38.1%). Patients who had two or more comorbidities were 98 patients (34.3%). Based on the severity of the subject, 55 patients were categorized as mild (19.2%), categorized as moderate in 62 patients (21.7%), and categorized as severe as 116 patients (40.5%). At the same time, the subject categorized as critical was 53 patients (18.6%). Based on the length of stay, the patients treated for 1–7 days were 92 patients (32.2%), treated for 8–14 days were 134 patients (46.7%), and treated for 15 days were 60 patients (21.1%). Based on signs and symptoms, fever was 206 patients (72%), cough and runny nose were 214 patients (74.8%), *Gastroenterological involvement* was 33 patients (11.5%), *Respiratory involvement* was 248 patients (86.7%), and *Olfactory dysfunction* in 12 patients (4.2%). Based on the mortality rate, 37 patients (13%) died and 249 were alive (87%). The characteristic patients are shown in Table 1.

Table 1: Clinical and virological characteristics of the study population

Characteristics	Number of Patients (n=286)	Percentage (%)
Age (years)		
≥17	4	1.4
18–40	46	16.08
41–60	155	54.19
>61	81	28.32
Gender		
Female	132	46.2
Male	154	53.8
Signs/symptoms		
Fever on admission	206	72.03
Complaint of the upper respiratory tract	214	74.83
Shortness of breath	248	86.71
Gastroenterological involvement	33	22.54
Bronchopneumonia	123	43.01
Comorbid		
Diabetes Mellitus	99	34.61
Hypertension	92	32.16
COPD/Asthma	2	0.7
Heart disease	20	7
Malignancy	1	0.35
Others	109	38.11
Comorbid ≥2	98	34.2
Severity level		
Mild	55	19.2
Moderate	62	21.7
Severe	116	40.6
Coma	53	18.5
Mortality		
Death	37	12.94
Alive	249	87.06
Long of Hospitalization		
1–7 days	92	32.17
8–14 days	134	46.85
≥15 days	60	20.98

COVID-19 outcomes according to SARS-CoV-2 gene expression

The results of the ANOVA test for the SARS-CoV-2 E gene expression at four levels of severity showed significant differences, and further tests using the Tukey test showed significant differences in gene expression in all groups except the moderate group and mild (Figure 1).

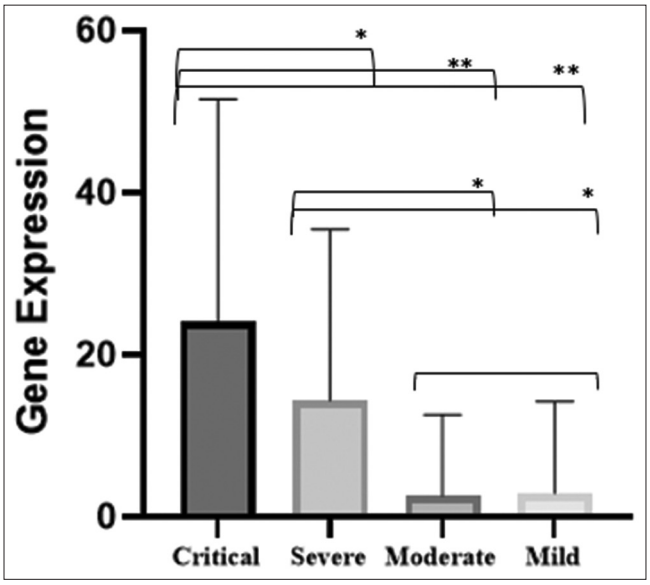

*Figure 1: Relative expression of the SARS-CoV-2 E gene in the Severity Group. * = Significantly different, (p < 0.05); ** = Significantly different (p < 0.001)*

The results of the Spearmen correlation test showed that there was a correlation between relative gene expression levels and the severity of COVID-19 patients (with r = 0.452 and p < 0.0001) (Table 2).

Table 2: The Relationship between SARS-CoV-2 Genes E Expression Levels with COVID-19 Patient Severity

			Genes E	Severity
Spearman's rho	Genes E	Correlation Coefficient	1	0.4525
		Sig. (2-tailed)	< 0.0001	< 0.0001*
		N	227	227
	Severity	Correlation Coefficient	0.4525	1
		Sig. (2-tailed)	< 0.0001	< 0.0001*
		N	227	227

*Spearman correlation test (p-value significant < 0.05). Severity: Mild, moderate, severe, critical.

The results of the ANOVA test for the SARS-CoV-2 N gene expression at four levels of severity showed significant differences, and further tests using the Tukey test showed significant differences in gene expression in all groups except the critical and severe groups the moderate and mild groups (Figure 2).

The results of the Spearmen correlation test showed that there was a correlation between relative gene expression levels and the severity of COVID-19 patients (with r = 0.374 and p < 0.0001). Gene expression relationship table with severity is shown in Tables 2 and 3.

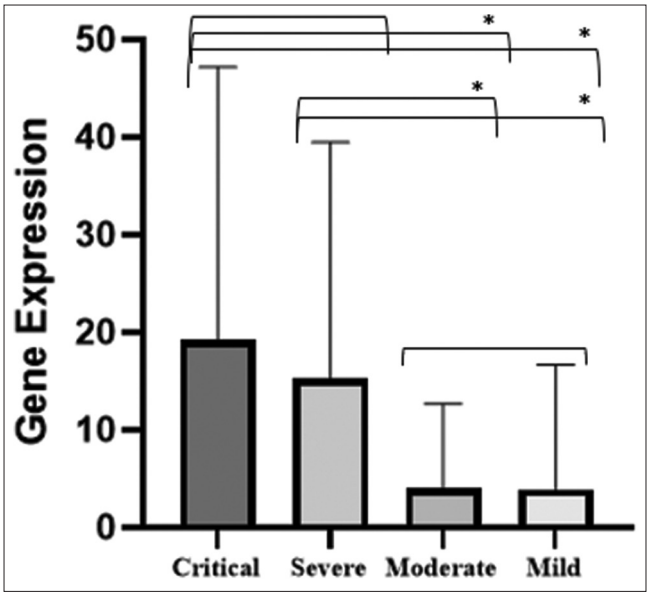

*Figure 2: Relative expression of the SARS-CoV-2 N gene in the Severity Group. * = Significantly different, (p < 0.05); ** = Significantly different (p < 0.001)*

Table 3: Relationship of SARS-CoV-2 Genes N Expression Level with Severity COVID-19 Patients Without Comorbid

			Gen N	Severity
Spearman's rho	Gen N	Correlation Coefficient	1	0.3741
		Sig. (2-tailed)	< 0.0001	< 0.0001*
		N	230	230
	Severity	Correlation Coefficient	0.3741	1
		Sig. (two-tailed)	<0.0001	<0.0001*
		N	230	230

*Spearman correlation test (p-value significant < 0.05). Severity: Mild, moderate, severe, critical.

Discussion

The first case of COVID-19 was found in Indonesia in March 2020. The incidence increased in mid-2020 and peaked in June 2021, when the Delta variant virus is expected to enter Indonesia in early 2021 [17]. In this study, data of COVID-19 patients were taken from the Regional General Hospital in West Java based on gender, age, comorbidities, symptoms, and severity. It is essential to understand the risk factors of a disease. The results showed that over 50% of COVID-19 patients at the West Java Hospital were female. This result is not the same as the research conducted in 2020 at the beginning of the pandemic in Indonesia that the proportion of male people living with COVID-19 was 59% and females were 41% [18].

A meta-analysis showed that men are 1.62 times more likely to have COVID-19 than women [19]. Other studies in Europe have shown that men are more at risk for contracting COVID-19 and with a higher mortality rate [20]. In Asia, excluding South Korea, the proportion of men who test positive for COVID-19 is higher than that of women. In South America and some European countries, more women test positive than men [18]. Differences in results across populations and places incidence and prevalence are not clearly defined. Random sampling from the population has not been carried out to determine the exact effect of COVID-19 to determine other risk factors such as gender, age, or comorbidities.

Based on the age classification, the results of the study show that patients with COVID-19 in West Java who are treated in hospitals with the most significant proportion are middle-aged 41–60 years, followed by elderly (60 years and over) and adults (18–40 years). The age of COVID-19 survivors is in the middle-aged group [21]. The study comes from a secondary dataset from the Nexoid United Kingdom shows that most patients covid-19 under the age of 40 years, followed by years of age 40–59 [22].

The results showed that the proportion of comorbid COVID-19 patients hospitalized at the West Java Hospital was hypertension and diabetes mellitus, with a ratio of almost 70% experiencing comorbidities of hypertension or diabetes or both. The results are in line with a meta-analysis conducted by Hong Liu that people with degenerative diseases such as hypertension, diabetes mellitus, heart disease have a 3.5-fold risk of having COVID-19 and a higher risk of death [19]. Chronic conditions of degenerative disease diabetes, hypertension, and Coronary Arterial Diseases/ Cardio Vascular Diseases, together with predisposing conditions alone, may be related to the etiology of the COVID-19 pathogenesis. Chronic diseases share some common features with the disorder and its complications, such as endothelial dysfunction, levels of pro-inflammatory cytokines, and alterations in the innate immune response [22].

The gene expression level of SARS-CoV-2 of COVID-19 positive patients was assessed by calculating the relative quantification by comparing the expression of the SARS-CoV-2 gene with the expression of the internal control gene (Formula $2^{-\Delta\Delta CT}$). The controls used in this calculation are patients with confirmed COVID-19 who are asymptomatic. The results showed that the expression levels of the SARS-CoV-2 gene varied greatly, so the standard deviation was very high. Figures 1 and 2 show the average expression of E and N gene in the critical, severe, moderate, and mild groups, respectively, which is 24.15 ± 27.38; 14.33 ± 21.17; 2.58 ± 9.98; and 2.88 ± 11.36. The average N expression in the critical, severe, moderate, and mild groups was 19.17 ± 28.04, respectively; 15.26 ± 24.20; 4.07 ± 8.59; and 3.95 ± 12.73.

The SARS-CoV-2 genome has similar sequencing characteristics to those of SARS-CoV and MERS-CoV. The human coronavirus is a positive sense (30 kb) RNA virus. Two types of proteins characterize human coronaviruses, structural (Spike [S], Nucleocapsid [N], Matrix [M], and Envelope [E]) and non-structural proteins (Nsp1 to Nsp16), including RdRp [6], [23].

The results showed a significant difference between the SARS-CoV-2 E and N gene expression in the

severity group. Furthermore, there was also a correlation between the expression of the SARS-CoV-2 E and N genes with the severity. This study is in line with research by Kaur *et al.* Patients with symptoms of olfactory taste disorder (OTD) had low CT values for genes E, N, and RdRp, which indicated a high viral load in these patients and influenced the occurrence of OTD symptoms in those patients [24]. The coronavirus encodes a protein Nsp1 that inhibits phosphorylation of signal transducer and activator of transcription 1 STAT 1, thereby inhibiting IFN-1 secretion. The low amount of IFN-1 triggers the emergence of pro-inflammatory cytokines in the patient's body. An increasing number of viruses causes an increase in the body's excessive production of cytokines, a condition known as cytokine release syndrome (CRS) or cytokine storm. This condition causes massive alveolar injury and multiple organ failures and leads to a fatal outcome [14], [25]. Studies have shown that plasma SARS-CoV-2 viremia has a clear association with clinical symptoms and disease severity, lower absolute lymphocyte counts, higher levels of inflammation, and risk of death [26]. Patients with leukocytosis and lymphopenia have a higher risk of developing severe and causing death. The occurrence of lymphopenia in severe disease may be due to lymphocyte apoptosis due to increased blood levels of cytokines in patients with severe disease [27], [28], [19].

In viral infectious diseases, initial viral load is often associated with disease severity [30]. However, this consensus on COVID-19 disease has not yet been agreed upon. Several factors affect the severity, length of stay, and mortality, namelyk male gender, age, and comorbidities such as hypertension, diabetes mellitus, congestive heart failure, kidney disorders, and immune system disorders [31], [32], [33]. Several studies have shown that epigenetic factors can influence the severity of patients, such as the expression of the ACE2 and dipeptidyl peptidase-4 (DPP4) genes [34].

The limitation of this study is that the CT of the PCR was gathered from medical records, not done by the researcher. It is not possible to use a single test for the entire observation period due to limitations of the test system, tools, or reagents. The laboratory examination method was developed by the hospital so that variations are very likely to occur. The interpretation of a single Ct value still has to be done with caution because it may be influenced by the sampling method, the gene being analyzed, testing how to determine the test performed, and analytical limits [14], [35], [36], [37].

Conclusion

This study shows a correlation between the level of expression of the E and N gene with the severity of patients. Therefore, it is suggested that further research be carried out using the cohort method on a larger sample and periodic RT-PCR examinations to see its effect on the course, severity, or improvement of COVID-19 disease.

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