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Independent Tendency of *ACE2* and *GRP78* Expression in SARS-CoV2 Infection

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Abstract: Severe acute respiratory syndrome-coronavirus 2 (SARS-CoV2) is a virus that attacks the respiratory tract and causes the COVID-19 pandemic. This virus utilizes the host receptor as a cellular entry. Angiotensin-converting enzyme 2 (*ACE2*) has been assumed to be the essential host receptor for SARS-CoV2 infection. Furthermore, another costimulatory molecule, such as glucose-related protein 78 (GRP78), has also been reported. However, there are several inconsistent clinical data that could be observed regarding these molecules' involvement during SARS-CoV2 infection. This study aims to observe the possible involvement of ACE2 and GRP78 during the infection phase through gene expression profile analysis. Clinical specimens used in this study were taken in positive and negative clinical samples after the standard swab sampling procedure from both oropharyngeal and nasopharyngeal swabs. Subsequently, nucleic acid samples were proceeded by conventional Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) to analyze the expression of *ACE2* and *GRP78*. Agarose gel electrophoresis was then performed before the densitometric analysis. Statistical analysis using Mann-Whitney Test and Independent Sample t-Test was applied to justify the gene profile difference between *ACE2* and *GRP78*. Our study suggested the enhancement tendency while they were not statistically significant in both *ACE2* and *GRP78* expression from the positive SARS-CoV2 samples.

Keywords: gene expression; *ACE2*; *GRP78*; SARS-CoV2

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has been a global issue these days. The disease with pneumonia-like characteristics was first reported in Wuhan, China, in December 2019. It was declared a pandemic in March 2020.1 Originally, it attacks the respiratory tract and causes acute respiratory disease with clinical manifestations such as fever, cough, shortness of breath, and myalgia.[2] Some other symptoms such as sputum production, headache, hemoptysis, and diarrhea are also reported.[3] Furthermore, the existence of collective symptoms could be assumed to a person whom SARS-CoV2.4 infects

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) as a cause of COVID-19 is a human Coronavirus (hCoV) that belongs to the betacoronaviridae family.[4] SARS-CoV2 is an enveloped virus and composed of several structural proteins, including membrane (M), envelope (E), nucleocapsid (N), and also spike (S).[5] Considering the viral genetic background, including its identity, SARS-CoV2 has been reported to have around 79,5% similarity in its nucleotide sequence against to the previous SARS-CoV which also has 96% similarity to the bat coronavirus.[6] Despite both nucleotide sequence similarities, SARS-CoV2 tends to be transmitted easily with less severity.[3]

Infection of SARS-CoV2 is initiated through the attachment of viral spike (S) protein to the responsible receptor in the host cell.[7] SARS-CoV2 receptor believed to be responsible for the infection is angiotensin-converting enzyme 2 (ACE2), which is then similar to the previous SARS-CoV receptor.[5,8] Further reports also

concerned the involvement of other molecules during SARS-CoV2 infection since there are some complexities in determining only a single causative related cell surface receptors.[9]

Structural analysis performed on SARS-CoV2 spike protein shows that this protein could also bind to the cell surface's glucose-related protein (GRP78).[9] Glucose-related protein (*GRP78*) is a chaperone molecule abundantly found in the eucaryote endoplasmic reticulum.[10] Normally, *GRP78* is located in the endoplasmic reticulum lumen area. However, when the cell is undergoing stress, this molecule escapes and translocates to the cell membrane. Thus, it is easily reached by the virus and initiates the viral infection.[11]

SARS-CoV2 can be detected in upper and lower respiratory tracts, including throat, nasal, nasopharyngeal, sputum, and bronchial fluid. Practically, a nasopharyngeal swab is a common procedure to obtain SARS-CoV2 containing specimens in combination with an oropharyngeal swab.[12] Recently, there has been a screening study reporting about gene expression patterns in both the upper and lower respiratory tract, which shows that *ACE2* is the most varied expression while *GRP78* is the highest level of expression in human respiratory epithelial cells.[8] Therefore, there are other possibilities for the involvement of other molecules in SARS-CoV2 infection besides the *ACE2* receptor.

This study was performed using SARS-CoV2 positive and negative nasopharyngeal and oropharyngeal swab specimens collected from RS Asri Medical Center Yogyakarta. It was purposed for the gene expression analysis in positive and negative SARS-CoV2 conditions to observe other functional viral receptors. Therefore, both gene expression discrepancies and alterations may suggest the direct involvement of *GRP78* along with *ACE2* during the SARS-CoV2 infection.

MATERIAL AND METHOD

This research was an observational analytic laboratory study and applied 26 positive samples(n=26) and 16 negative samples (n=16) following the sample size equation:

$$\frac{(Z_1-\frac{1}{2}\alpha)^2\ x\ p\ x\ (1-p)}{D^2} = \frac{(1{,}96)^2\ x\ 0{,}12\ x\ (0{,}88)}{0{,}1^2} = 41$$

The healthcare professionals collected the specimens from patients who came to the Asri Medical Center Hospital, Yogyakarta. It was provided by MMT Laboratory with the population, which ranged from November 2020 to January 2022 and had Ct value smaller than 25 for each of all three SARS-CoV2 target genes (*N, E,* and *RdRp*) that covered around 12% of the population. All samples were taken according to the consecutive blinded sampling principles. This study was conducted under the ethical approval of the Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta (No. 029/EC-EXEM-KEPK FKIK UMY/IV/2021).

The obtained specimens were further extracted by the laboratory technical support from the Molecular Medicine and Therapy (MMT) Laboratory. The RT reaction was performed by adding 6 µl RNA into the PCR tube as a total initial mixture. Then, RNA denaturation was performed using a thermocycler (Scilogex, USA) for 5 minutes at 65°C. Subsequently, 2 µl Mix A (gDNA Remove Solution) was added, then vortex and spindown for 2-3 seconds prior to gDNA removal for 5 minutes at 37°C. 2 µl 5X Master Mix II (Bioline, London, UK) was sequentially added to the mixture, then vortex and spindown for 2-3 seconds. The RT reaction was conducted under the conditions of 37°C for 15 minutes, 50°C for 5 minutes, and 98°C for 5 minutes.

The PCR amplification was proceeded by mixing 25 µl 2X My Taq HS Mix (Bioline, London, UK), 20 µl nuclease-free water (Bioline, London, UK), and 3 µl template cDNA previously prepared. 1 µl forward and reverse primer of each gene target was used.[13,14] PCR amplification steps were performed following the conditions of 95°C for 4 minutes, 94°C for 30 seconds, 42°C for 30 seconds, and 65°C for 30 seconds in 35 cycles. Followed by 65°C for 4 minutes and hold at 4°C for *ACE2*. Similar conditions were applied for *GRP78* with modification in cycling and extension as follows, 95°C for 30 seconds, 56°C for 20 seconds, and 72°C for 30 seconds in 36 cycles followed by 72°C for 4 minutes and hold at 4°C.

The PCR products were undergone electrophoresis with 1% agarose gel (Vivantis, Selangor, Malaysia), 1X TAE running buffer (Vivantis, Selangor, Malaysia), and 1 µl Gel Red (Biotium, California, USA). 2 µl novel juice (GeneDireX, USA) were added into 10 µl PCR product for loading preparation. 5 µl of ladder marker (Vivantis, Selangor, Malaysia) was subsequently loaded as an indicator marker. Electrophoresis analysis was performed for 20-30 minutes using MiniRun GE (Bioer, Hangzhou, China). The running result was analyzed

using a UV transilluminator (GeneDireX, USA) and a 48-megapixel mobile camera Galaxy A51 (Samsung, Seoul, Korea) for image detection. The expressions of signals were then quantified under densitometric analysis using ImageJ version 1.52 (Wayne Rasband, National Institute of Health, USA). The data were then analyzed with Shapiro-Wilk for data normality test and followed by Independent Sample t-Test for the parametric data and Mann-Whitney Test for the nonparametric data using SPSS version 20.0 (IBM, New York, USA).

RESULT

The expression profile of *ACE2* and *GRP78* in each sample is shown in Figure 1 to Figure 6. *β-actin* was considered an internal control. Normality statistical data indicated that *ACE2* data were not normally distributed (p 0.049 for positive & p 0.048 for negative, $p<0.05$), while *GRP78* data were normally distributed (p 0.248 for positive & p 0.067 for negative, $p>0.05$).

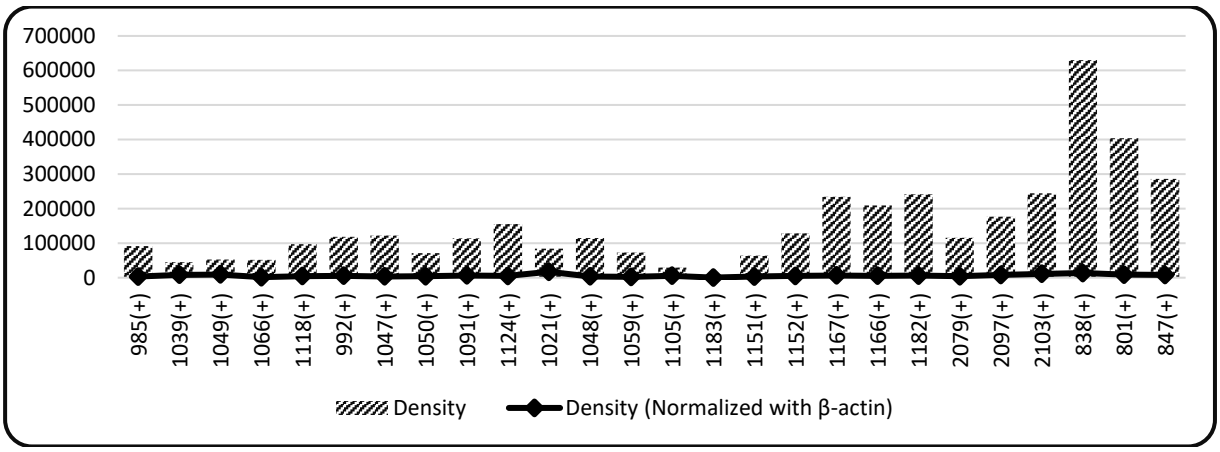

Figure 1. The density of *ACE2* in Positive Samples

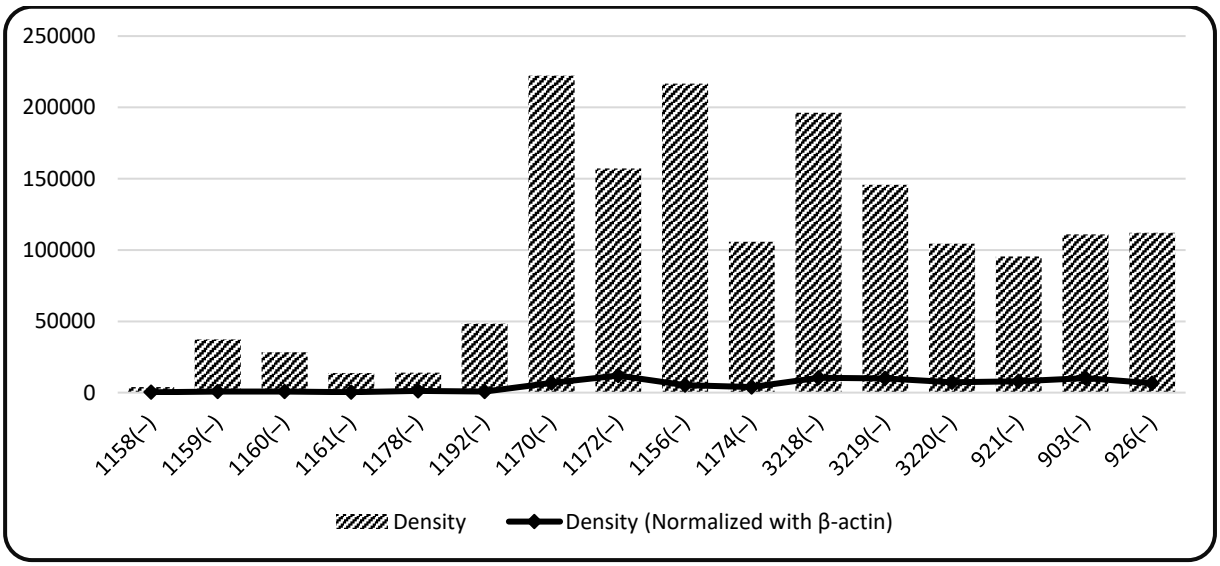

Figure 2. The density of *ACE2* in Negative Samples

Figure 1 and Figure 2: Each graphic bar indicated the individual independent signal density of the *ACE2* gene in both positive and negative clinical samples, respectively, at 238bp, while the black line within the bars indicated the normalized density signal toward each correspond *βactin*

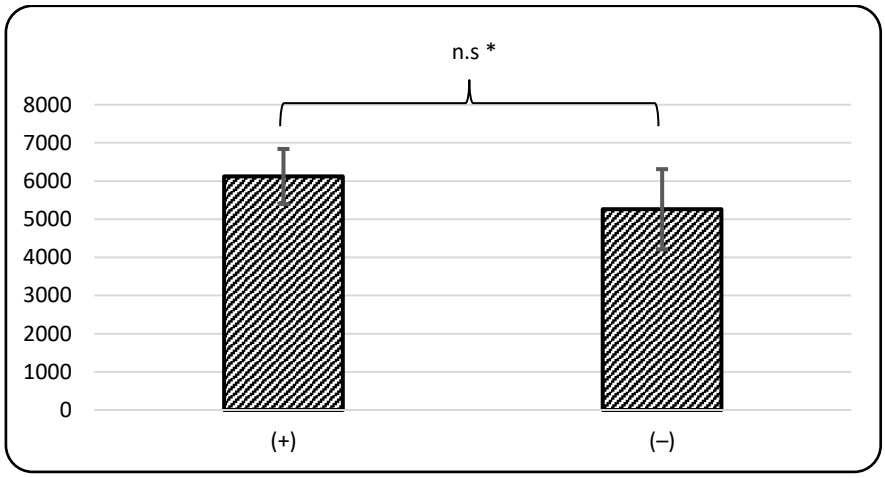

Figure 3. The Mean Density of *ACE2*

Figure 3: The graph shows a comparison between the average expression of *ACE2* in positive and negative conditions of SARS-CoV2. The average expression of *ACE2* in positive conditions is higher than in negative conditions, with standard error in positive conditions also higher than in negative conditions. Statistical analysis showed that *ACE2* expressions in positive and negative conditions are not significantly different. *p > 0.05

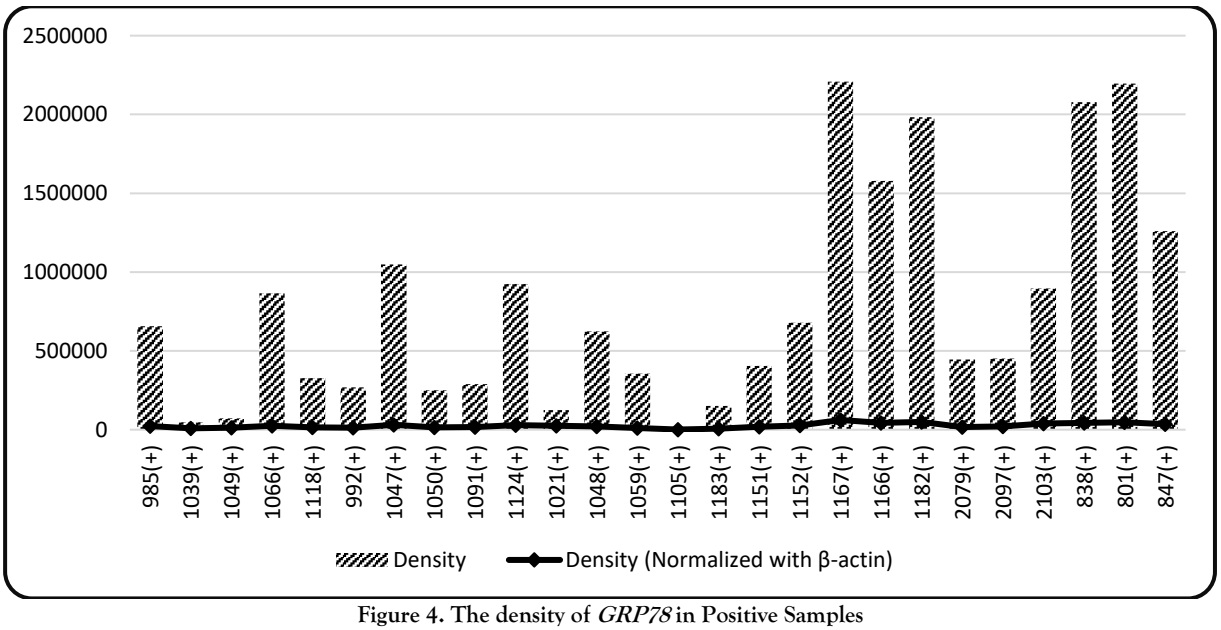

Figure 4. The density of *GRP78* in Positive Samples

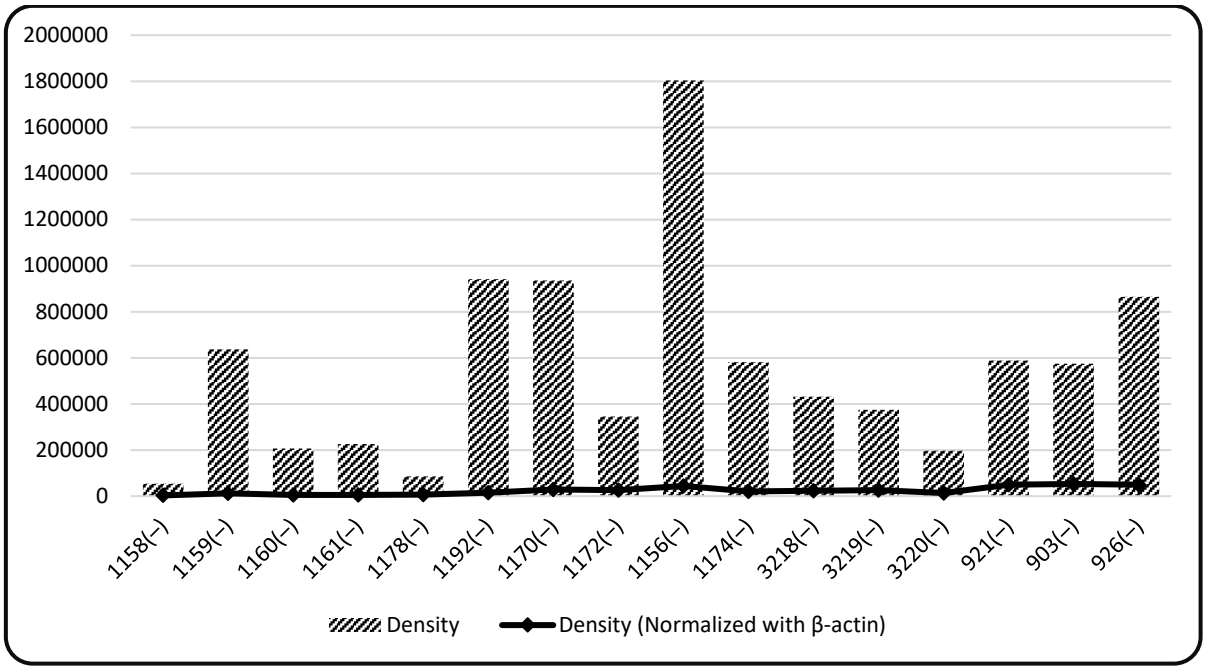

Figure 5. The density of *GRP78* in Negative Samples

Figure 4 and Figure 5: The bar graph shows the density of *GRP78* in both the positive sample (n=26) and the negative sample (n=16) at 278bp. Meanwhile, the histogram graph shows the GRP78 density of each sample normalized with *βactin*.

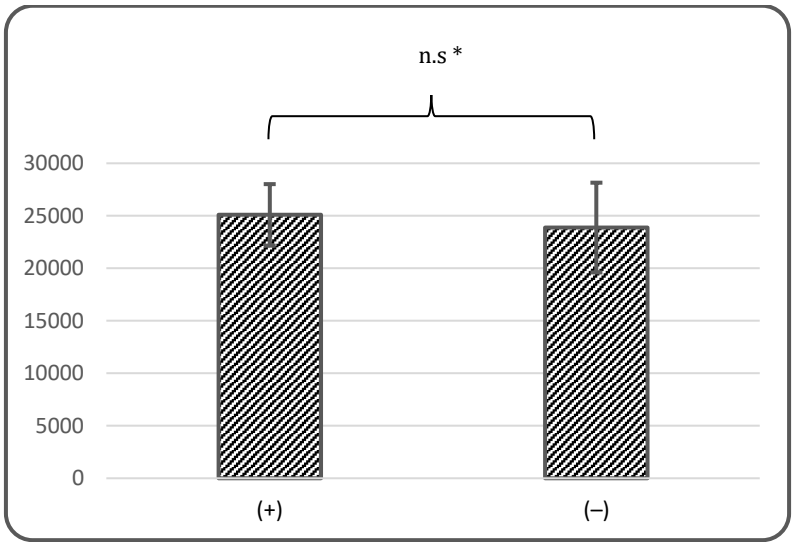

Figure 6. The Mean Density of *GRP78*

Figure 6: The graph shows a comparison between the average expression of *GRP78* in positive and negative SARS-CoV2. The average expression of *GRP78* in positive conditions is higher than in negative conditions, while standard error in positive conditions is also higher than in negative conditions. Statistical analysis showed that the expression of *GRP78* under positive and negative conditions was not significantly different. *p > 0.05

The signal density showed the gene expression of *ACE2* and *GRP78*, which could be observed at 238bp and 278bp, respectively. The higher the density is, the higher the expression accordingly will be. Statistical analysis of *ACE2* gene expression had a *p*-value of 0.489 (p>0.05), indicating no significant difference between both positive and negative samples. Similarly, no significant difference could be observed in GRP78, which had a p-value of 0.811 (p>0.05).

In the positive group, the mean density of *ACE2* was 6121.77 with a standard of error of 719.572, while the negative group showed 5261.93 with a standard of error of 1049.489. As for *GRP78*, the positive group

showed 25085.79 with a standard error of 2931.446, while the negative group was 23879.62 with a standard error of 4275.094.

DISCUSSION

This study showed relative gene expression related to *ACE2* and *GRP78*, which were previously reported as a part of responsible genes in COVID-19 infection. Since our data showed quite similar expression levels in both samples, it suggested that the SARS-CoV2 infection had no effects on the expression level of *ACE2* and *GRP78*. However, the result also suggested an enhancement tendency in the SARS-CoV2 positive samples, as shown in Figure 3 and Figure 6.

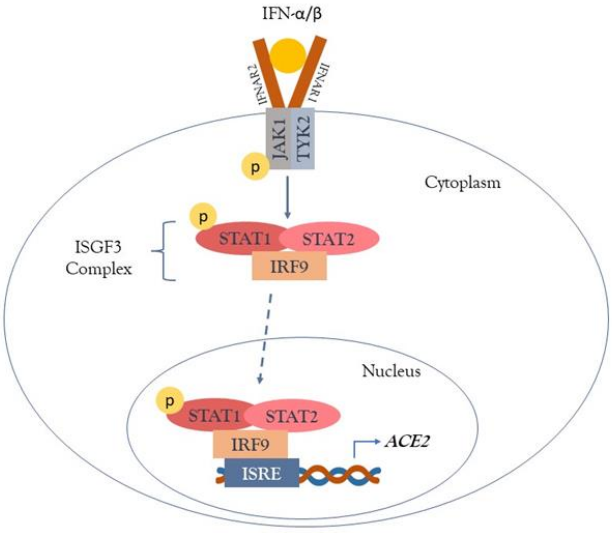

Figure 7. JAK/STAT Signaling Pathway (Modified from Lodhi et al. (2021))

JAK1: Janus Kinase 1. TYK2: Tyrosine Kinase 2. STAT1: Signal Transducers and Activators of Transcription 1. STAT2: Signal Transducers and Activators of Transcription 2. ISGF3: Interferon Stimulating Gene Factor 3. IRF9: Interferon Related Factor 9. ISRE: Interferon Stimulating Regulatory Elements.

These results align with Gutiérrez-Chamorro et al. study, which reported that the *ACE2* expression was not significantly different in both positive and negative SARS-CoV2.[15] However, a study by Zhuang et al. stated that *ACE2* had an enhancement in SARS-CoV2 patient.[16] This disagreement in the *ACE2* expression profile could be related to the multisignaling pathway in the upstream mechanism of ACE2 promoter activity, such as the Interferon-Stimulated Gene (ISG) complex. In general, interferon is a host antiviral responsive molecule that can be induced by viral infection. The enhancement of interferon expression, particularly type I, may increase *ACE2* expression through the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway. Janus Kinase 1 (JAK1) and TYK2 will phosphorylate STAT1 and STAT2, which subsequently form IFN-Stimulated Gene Factor 3 (ISGF3) complex. This complex may access the Interferon Stimulating Regulatory Elements (ISRE) on the ACE2 promoter region, thus controlling *ACE2* expression. Regarding the cell environment signaling, this mechanism may lead to the enhancement of *ACE2* regulation, as seen in Figure 7.[17]

Furthermore, similar to *ACE2*, *GRP78* also showed a similar tendency of enhancement in the positive samples. In the positive samples, SARS-CoV2 replicated in the host cell using host translational machinery, subsequently interfering endoplasmic reticulum and inhibiting protein synthesis from producing viral proteins. This condition could lead to endoplasmic reticulum stress (ER stress).[18] According to Carlos et al., ER stress activates the unfolded protein response (UPR) in the endoplasmic reticulum and enhances the *GRP78* expression level.[19] The enhancement tendency in both genes expression in positive samples could be observed through the biological traits of the data, following the data distributions and central tendency since the value of standard error would be reduced by increasing the number of data.[20]

The non-significant expression difference from our data was also consistent with the previous result by Aguiar et al., demonstrating the most expressed genes in the upper respiratory tract, *GRP78* and *ACE2*, in

the SARS-CoV2 positive condition. This condition also happened in the lower respiratory tract. Furthermore, Chaudry et al. also explained that *ACE2* expression in the upper respiratory tract was lower than in the lower respiratory tract. *ACE2* expression is commonly abundant to be found in type II alveolar cells. Meanwhile, *GRP78* is most abundantly expressed by bronchus, bronchial, and alveolus cells.[11] Although the oropharynx and nasopharynx are not ideal sites to evaluate precisely the expression of *ACE2* and *GRP78* in SARS-CoV2 infection, the specimen collection for SARS-CoV2 detection was performed by oropharyngeal and nasopharyngeal swab method.

Furthermore, this study's limitations include the fewer samples analyzed and the possible variation during preanalytical procedures. However, the analyzed samples were also normalized with human *β-actin* as a housekeeping gene.

CONCLUSION

Based on the result of this study, it can be concluded that the expression of *ACE2,* as the putative recognized SARS-CoV2 functional receptor, and the *GRP78* as a potential co-receptor for SARS-CoV2 infection, tended to be independent of the SARS-CoV2 existence. Meanwhile, it also needs to consider the cellular and tissue environment signaling condition since there was an enhancement profile.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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