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Original Article

Correlation of COVID-19 Receptors with Neutrophils and Their Role in the Adherence of Co-Infected Bacteria

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Abstract

The COVID-19 caused by the SARS-CoV-2 virus has an impact on all aspects of patient care. Since the onset of this disease pandemic in 2019, numerous studies have been published which have attempted to identify virus receptors in the upper respiratory tract, such as nasal, oropharynx, and lung and their role in coinfection of bacterial adherence. In this study, the level of m RNA for platelet-activating factor receptor (PAF-R) and angiotensin-converting enzyme 2 receptor (ACE2-R) were detected in the whole blood of COVID-19 patients and controlled by using real-time reverse transcription-polymerase chain reaction technique. The results of the expression level of the PAF-R gene were higher in patients (43 ± 12.5) than in the healthy control (40 ± 2.1). Moreover, the expression level of ACE2-R was significantly (0.0001) increased in patients (27.5±6.2), compared to the control group. In addition, there was an elevation of neutrophils (79.6±17.6%) and PAF-R level (43%) in COVID-19 patients in comparison to the control (40) with a positive correlation between these factors (r=0.8769, P=0.0001). Nasopharyngeal epithelial cells showed a higher adherence rate (86%) to both bacteria isolates (*Streptococcus pneumonia* and *Staphylococcus aureus*) in patients than in the control group. Increased expression of PAF-R and ACE2-R genes in COVID-19 patients and co-infected bacteria disease could be the factors for the SARS-CoV-2 virus to enter the cells of the host.

Keywords: ACE2-R, COVID-19, Neutrophil, PAF-R, RT-PCR, *Staphylococcus aureus*, *Streptococcus pneumonia*

1. Introduction

The coronavirus disease 2019 (COVID-19) was induced by SARS-CoV-2 infection and has high mortality and morbidity rates. The main complication of COVID-19 is respiratory dysfunction, including diffused alveolar damage and respiratory failure. A few recent studies have demonstrated that the angiotensin-converting enzyme 2 receptor (ACE2-R) and platelet-activating factor receptor (PAF-R) genes level was significantly unregulated in the pharyngeal epithelium of patients with upper respiratory tract infection (URI). These studies have hypothesized that the higher risk of coinfection with coronavirus disease 2019 (COVID-19) is due to the patient's differential expression of ACE2, the receptor that SARS-CoV-2 uses for host cell entry (1, 2).

Previously, ACE2 was identified as a SARS-CoV and HCoV-NL63 entrance receptor, considering that the SARS-CoV-2(COVID-19) sequence is identical to SARS-CoV; hence, ACE2 seems to be used by SARS-CoV-2 cell input receptor. The ACE2 is expressed in several tissues, including the renal, cardiovascular, and gastrointestinal tissues (3, 4). Based on the results of a recent study by Khaddour, Sikora (5) ACE2 and its receptor is an interferon-stimulated gene in the human respiratory system, epithelial cells, which may explain the increasing coinfections with COVID-19 virus and

other respiratory pathogenic bacteria significantly by increasing the adherence rate of this pathogens.

The other receptor was PAF which was one of the potent phospholipid mediators with a wide range of bioactivity and has been shown to increase the binding of pathogenic bacteria to pharyngeal epithelial cells (6). A high number of PAFs are generated from the endothelial cells and stromal cells included neutrophils, eosinophils, basophils, and platelets (7). The biological action of PAF is mediated by a particular G protein couple receptor (PAFR), found in numerous hemopoietic cells, including platelets, neutrophils, monocytes, and dendritic cells, in the membrane of cells (3).

The present study aimed to analyze ACE2-R and PAF-R gene expression in the whole peripheral blood of patients with COVID-19 patients as well as the rate of bacterial adherence in the epithelial cell as a co-infection.

2. Materials and Methods

2.1. Sample Collection

This case-control study was performed from October 1, 2020, to February 1, 2021. In total, 250 (146 male and 104 female) samples were collected from COVID-19 patients. All of the patients had positive results to real-time reverse transcription-polymerase chain reaction (RT-PCR) nasopharyngeal swab for COVID-19. The clinical signs were screened and recorded in Al-Najaf AL-Ashraf National center for COVID-19 in the first week of infection which included fever, loss of smell and taste, headache, and nasal congestion (based on World Health Organization guidelines) (8). In total, 100 healthy individuals were included in this study as the control group. The median age was 51 years and the age range was 7-82 years old. For the purposes of the study, 10 ml of fresh venous blood samples were collected from COVID-19 infected patients with sterile syringes in the morning between 8:00 and 9:00 am after an overnight fast. Afterward, 3 ml of blood samples were put in a plain tube and allowed to clot then centrifuged at 4000 rpm for 10 min. It was used for the detection of the serum level of some cytokines by using the ELISA technique. It should be mentioned that 2 ml were collected in EDTA tube for lymphocyte detection by using 5 differential automated devise (Mindry, China) for complete blood count for white blood cell differential as neutrophil one of these cells. The other 5 ml were put in an EDTA tube for gene expression analysis of PAF-R and ACE2-R.

Nasopharyngeal swabs were taken from each patient to detect the presence of co-infected bacteria. All swabs were cultured on blood agar and mannitol agar for detection and isolation of *Streptococcus pneumonia* and *Staphylococcus aureus*. VITEK-2 compact system used GP cards which contained 43 biochemical tests and one negative control well.

2.2. Gene Expression Analysis of Platelet-Activating Factor Receptor and ACE2-R

2.2.1. RNA Isolation and Reverse Transcription Step (RT-PCR)

Total RNA was extracted from whole blood (5 ml collected in an EDTA tube) by using the ELK biotechnology kit (China) according to the protocol provided by the manufacturer. The RNA samples were stored at -20 until use. The concentration of RNA was determined by measuring its absorbance at 260 nm (A260-A280). The (A260/A280) ratio greater than 1.6 was accepted.

A reverse transcriptase kit (High-Capacity cDNA Reverse Transcription Kit, UK) was used for complementary DNA (cDNA) on a 2720 thermal cycler (China). For cDNA synthesis, RNA (10 µl) was reverse transcribed in a final volume of 20 µl containing 1 µl of reverse transcriptase enzyme, 2 µl of 10X RT random primer, 2 µl of 10X RT buffer, 0.8 µl 25X DNTP mix, and 4.2 µl of nuclease-free water. The samples were incubated at 25 ˚C for 10 min (primer annealing), and at 42 ˚C for 15 min (reverse transcription). Reverse transcriptase was inactivated by heating at 85 ˚C for 5 min. All products were stored at (-20 ˚C) till used in the next step.

2.3. Normalization and cDNA Amplification

For cDNA amplification, relative quantitation of (PAF-R and ACE2-R) mRNA expression was normalized to the endogenous reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by real-time reverse transcription-polymerase chain reaction.

The PCR reaction mixture (final volume, 20 µl) contained 0.4 µl of each primer according to table 1 (GDS/Bio), 10 µl of 2x q PCR SYBR Green Master Mix Lo (China), 4.2 µl of nuclease-free water, and 5 µl of cDNA. Thermocycling conditions were 3 min at 95 ˚C, followed by 40 cycles at 95 ˚C for 10 sec, 60 ˚C for 10 sec, and 72 ˚C for 20 sec. For relative quantification of the results obtained by RT-PCR, the comparative cycle threshold method was used. Analysis was performed using MXpro3005 software (version 2.0.1).

Table 1. Sequence of primers of Platelet-activating factor receptor (PAF-R), angiotensin-converting enzyme 2 receptor (ACE2-R), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Primer name	Sequence of primer
PAF-R	5’ –CGGACATGCTCTTCTTTGATCA-3’ (foreword), 5’ -GTCTAAGACACAGTTGGTGCTA-3’ (reverse),
ACE2-R	5’- GTGATCCCATGGCTACAGAGG -3’(foreword), 5’-CTTGGGTTGGGCGCTATTCA -3’ (Reverse)
GAPDH	5’-GTCTCCTCTGACTTCAACAGCG -3’(foreword) 5’-ACCACCCTGTTGCTGTAGCCAA -3’ (reverse)

2.4. Bacterial Adherence Assay

The rate of adherence of pathogenic bacteria isolated from COVID-19 patients as co-infected bacteria to oropharyngeal epithelial cells of patients and healthy control was calculated according to LOMBERG, DE MAN (9). Attached bacteria on 50 successive cells were counted and the degree of adhesion was expressed as the number of bacteria attached per cell.

2.5. Statistical Analysis

In this study, data were analyzed statistically by SPSS for Windows (version 17.0; SPSS Inc., USA). The Mann-Whitney U-test and Spearman’s correlation were used for statistical analysis and determined with a significant level of ≤ 0.05.

3. Results and Discussion

In this study, the expression levels of PAF-R and ACE2-R mRNA in the whole blood were analyzed in COVID-19 patients. The RT-PCR was used to determine the expression of these receptor genes.

In figure 1, the melting temperature is uniform with sharp peaks, which indicates that PAF-R and ECA2 R, and GAPDH are the only amplicons in the amplification products.

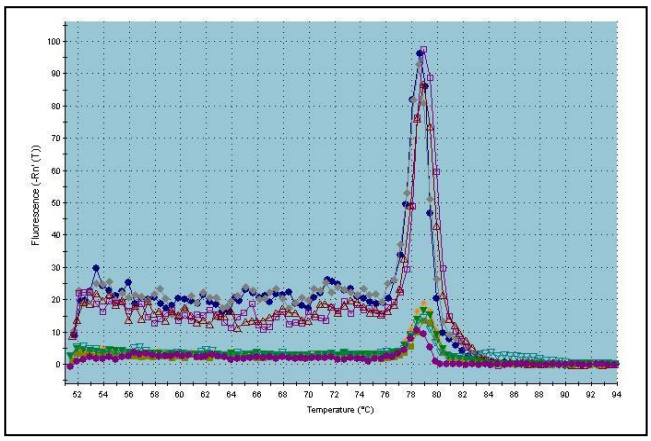

Figure 1. Melting curves of platelet-activating factor receptor and ECA2-R mRNA and Glyceraldehyde 3-phosphate dehydrogenase mRNA

The expression level of the PAF-R gene was higher in patients (43±12.5) than in the healthy control group (40±2.1). Moreover, the expression level of ACE2-R was significantly (0.001) increased in patients (27.5±6.2) than in the control group as shown in figure 2.

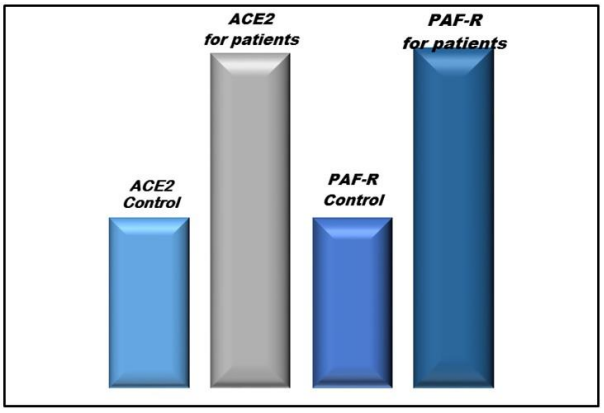

Figure 2. Level of expression gene of platelet-activating factor receptor and angiotensin-converting enzyme 2 receptor in COVID-19 patients and control group

Elevation of the level of neutrophils and PAF-R showed that in figure 3, the level of neutrophils was determined (79.6) and PAF-R (43). A positive correlation between neutrophils and PAF-R was observed (r= 0.8769, P= 0.0001) in COVID-19 patients as in figure 4.

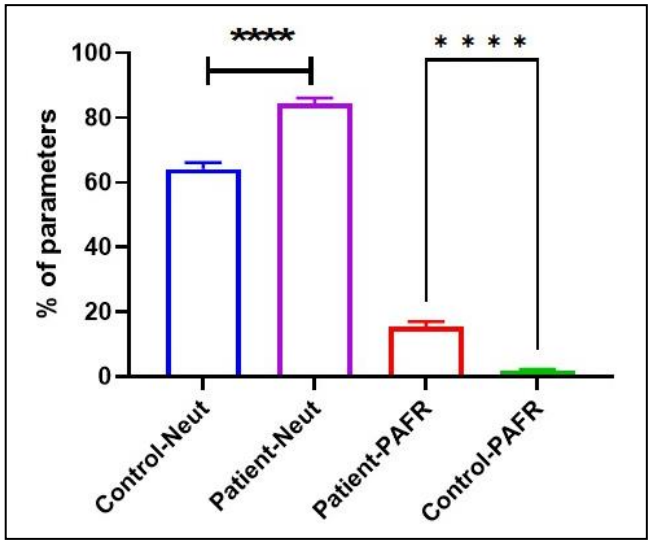

Figure 3. Relationship of platelet-activating factor receptor and Neutrophil cells in COVID-19 patients

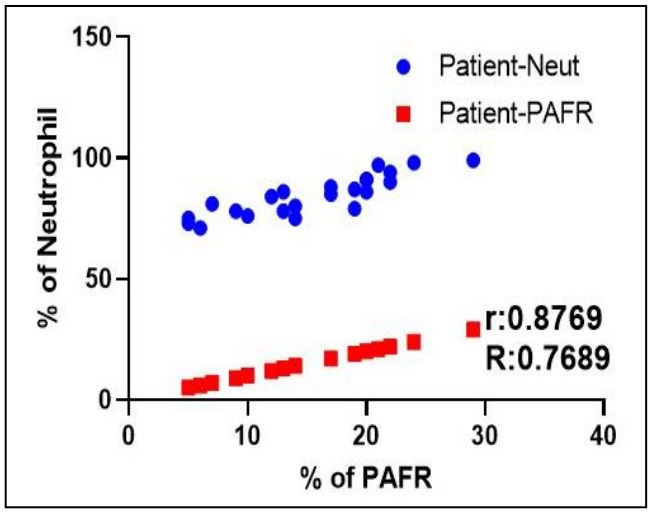

Figure 4. Correlation of platelet-activating factor receptor and Neutrophils in COVID-19 patients

Previously, ACE2 was identified as an entry receptor for SARS-CoV and HCoV NL36. Analysis of the genome of COVID-19 showed that this new virus shares about 80-90% sequence identity with the original SARS-CoV (10). Both bioinformatics modeling and *in vitro* experiments indicated that 2019-nCoV was likely to utilize ACE2 as a receptor to enter human cells. It has been found that COVID-19 is able to bind to the ACE2 receptor on the surface of epithelial cells. It has also been confirmed that ACE2 is essential for the infection of cells by COVID-19 (11). Results of a recent study have reported that ACE2 is an interferon-stimulated gene in human airway epithelial cells that leads to an increase in concomitant infections with COVID-19 and other respiratory pathogens (2).

Results of the present study, which were consistent with those of the study performed by Rockx, Baas (12), showed that ACE2 gene levels were significantly increased in the blood of patients with COVID-19 and were significantly positively correlated with age and body temperature. Zhou, Yang (13) showed that ACE2 was found in all of the samples, including those of the healthy population and patients with chronic airway diseases. Therefore, there may be no significant difference between a healthy population and patients with chronic respiratory diseases in terms of susceptibility to COVID-19 infection. Previous studies have found that ACE2 is related to the severity of acute respiratory syndrome induced by the SARS-CoV2 infection, and it mediates the production of cytokines associated with acute respiratory distress syndrome (14) (Figure 4).

According to the evidence, ACE2 is the main receptor of the COVID-19 host cell and plays an important role in the entry of the virus into the cell to cause the final infection. The results of this study also showed that the high expression of ACE2 increased the expression of genes involved in viral replication, which may enhance the ability of the virus to enter the host cells. McGonagle, Sharif (14) supported the hypothesis that the transcriptome of epithelial cells is altered after infection by COVID-19 through increased ACE2 expression, which is beneficial to the replication and assembly of the virus as well as the entry of the virus into host cells.

Significantly, the regulatory transformation of receptors has been reported after upper airway

infection, including PAFR. The PAFR expression can be regulated by a variety of physiological and pharmacological factors and may vary with differentiation and activation status (1, 15).

In the study of concomitant respiratory infections in patients with COVID-19, the ability of *S. pneumonia* and *S. aureus* to adhere to nasopharyngeal epithelial cells in COVID-19 patients and healthy control were detected in this study according to Svanborg Eden, Larsson (16). It was found that the adherence rate of *S. pneumonia* (95) and *S. aureus* (77.5) to epithelial cells of patients was higher than the adherence rate of the same bacterial isolates to the nasopharyngeal epithelial cell of non-infected patients (19, 17.5, respectively) as shown in figure 5. This increase in adherence may be due to the increased receptor level of these bacteria on nasopharynx epithelial cells during infection. The results of this study showed that expression of ACE2 and PAF-R genes increased in COVID-19 and played an important role in the binding and entry of the pathogen into the cells of the patient and are also effective in concomitant bacterial infections in COVID-19 patients.

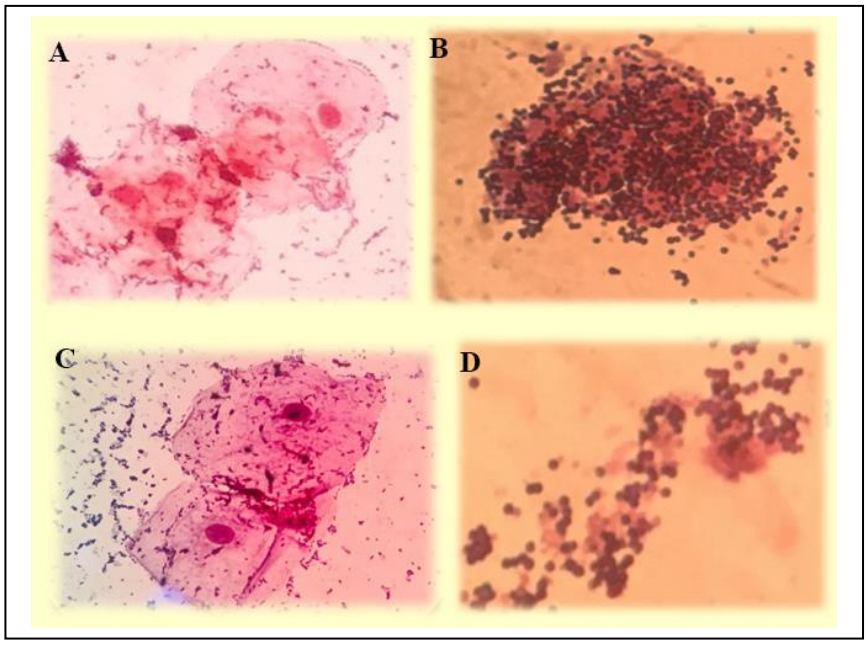

Figure 5. Adherence of *Bacterial isolates* to nasopharyngeal epithelial
(A) *Streptococcus pneumonia* to **(B)** and **(C)**. *Staphylococcus aureus* to **(D)**

Authors' Contribution

Study concept and design: R. A. N. S.
Acquisition of data: H. A. A. A.
Analysis and interpretation of data: H. A. A. A.
Drafting of the manuscript: R. A. N. S.
Critical revision of the manuscript for important intellectual content: R. A. N. S.
Statistical analysis: H. A. A. A.
Administrative, technical, and material support: R. A. N. S.

Ethics

This study was approved by the Ethics Committee of the Faculty of Science, University of Kufa, Kufa, Iraq, and informed consent was obtained from all participants.

Conflict of Interest

The authors declare that they have no conflict of interest.

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