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Upregulation of FOXP3 is associated with severity of hypoxia and poor outcomes in COVID-19 patients

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ABSTRACT

The levels of messenger RNA (mRNA) transcription of FOXP3, IFN-γ, TNF, IL-6 and COX-2 from both COVID-19 infected and control subjects were evaluated using SYBR™ green real-time polymerase chain reaction (RT-PCR). Severe/critical cases showed significantly lower lymphocyte counts and higher neutrophil counts than the mild or moderate cases. There were significantly lower levels of mRNA expressions of IFN-γ, TNFα and FOXP3 in COVID-19 patients than in the control group. On the other hand, IL-6 and COX-2 expressions were significantly higher in patients suffering from severe disease. FOXP3 expressions were correlated with the severities of hypoxia and were excellent in predicting the disease severity. This was followed by the IL-6, COX-2 and TNFα expressions. FOXP3 expression was the only biomarker to show a significant correlation with patient mortality. It was concluded that SARS-CoV-2 infection is associated with the downregulation of FOXP3 and upregulations of IL-6 and COX-2.

1. Introduction

In December 2019, SARS-CoV-2, a new member of the family Coronaviridae, emerged in Wuhan, China. The virus resulted in a harsh pandemic with more than 203,000,000 laboratory-confirmed cases and more than 4,3 million deaths worldwide as of August 11, 2021. Although the majority of patients who get the coronavirus disease 2019 (COVID-19) have good prognoses, 5–10% develop critical symptoms that may require mechanical ventilation and have fatal consequences (Guan et al., 2020; Hadjadj et al., 2020; Lai et al., 2020).

The immune system adopts different mechanisms in response to various pathogens. During the immune response, several inflammatory pathways are activated; however, an exaggerated response can cause a severe and sometimes uncontrolled inflammatory reaction (Dinarello, 2000; Hadjadj et al., 2020; Jamilloux et al., 2020). Pro-inflammatory cytokines, including interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumour necrosis factor (TNF) and gamma interferon (IFN-γ) (Dinarello, 2000), are important in the development of innate antiviral immune responsiveness. However, exaggerated responses may lead to severe inflammatory reactions. IL-1β induces the synthesis of cyclooxygenase-2 (COX-2) with a subsequent increase in prostanoid production that induces inflammation (Duque et al., 2006). IL-6 mainly induces pro-inflammatory signalling and modulates massive cellular processes, and it has been found to be associated with many viral diseases, inflammatory diseases and multiple cancers (Bruzzese and Lazzarino, 2020; Luo and Zheng, 2016). Homeostasis of the immune responsiveness is controlled by the regulatory T cells (Tregs) that work through downregulations and suppression of the host immune responses. The forkhead box P3 (FOXP3) protein is the gene expressed on the surface of Tregs (Bacchetta et al., 2007; Sakaguchi et al., 2010). The latter play an important role in the suppression of different inflammatory, allergic and autoimmune disorders, including pulmonary infections (Adamszik et al., 2019).
A cytokine storm results from a sudden increase in the pro-inflammatory cytokines with subsequent chemotaxis of macrophages, neutrophils and lymphocytes to the site of infection. The concentrations of some of these cytokines in the blood allow the discrimination between mild, moderate and severe cases (mainly IL-1β, IL-1Ra, IL-6, IL-7, IL-10, IP-10, IFN-γ and TNF-α) (Jamiloux et al., 2020). During moderate COVID-19 infection, the immune responsiveness reacts normally to viral infection in a robust manner. In contrast, with severe COVID-19 infection, the complications occur as a sequel to dysregulated and excessive immune responses (Ye et al., 2020). The innate immune response is exaggerated in an uncontrolled manner, while the adaptive immunity is impaired with subsequent deterioration of pulmonary functions due to severe tissue damage (Chen et al., 2020; Tan et al., 2020; Thevarajan et al., 2020). The exaggerated immune responses that lead to pulmonary tissue damage in patients with severe COVID-19 disease are still obscure. Most patients who are critically ill initially show only mild symptoms. However, their conditions deteriorate suddenly 9–12 days after the first onset of symptoms and sometimes during the process of recovery when the patients develop acute respiratory distress syndrome (ARDS) and multiple-organ failure, which may be followed by death within a short period of time (Hadjadj et al., 2020).

Although several studies have been published about the pathogenesis of COVID-19, our knowledge regarding the immunological features and the molecular mechanisms involved with respect to COVID-19 severity is still limited (Hadjadj et al., 2020). Understanding the pathogenesis of this severity is the first step toward designing therapeutic interventions that can prevent the progression of COVID-19 infection and save the patients’ lives (Ye et al., 2020). We aim in this study to evaluate the levels of expression of five genes; namely, FOXP3, IFN-γ, TNF, IL-6 and COX-2, in adults who are diagnosed with COVID-19 and to correlate these levels of expression with disease outcomes and clinical and laboratory data, especially those known to have prognostic value.

2. Methods

2.1. Ethical approval and consent to participate

All patients and control subjects signed informed consents, and the study was approved by the ethical committee of the National Cancer Institute (NCI), Cairo University. Clinical, radiological and laboratory data were collected from patients’ files.

2.2. Patients

This study included 111 hospitalized patients who were admitted to Cairo University hospitals. All patients presented to the hospitals between April and July 2020. The control group included 32 age-matched normal subjects. Sample size was calculated using SigmaPlot software 12.5.0.38 for Windows (SigmaPlot, Systat Software Inc. UK, 2011). Infection with the SARS-CoV-2 virus was confirmed by real-time polymerase chain reaction (RT-PCR) through mixed throat and nasal swabs.

2.3. Clinical and laboratory investigations

Patients were classified into mild, moderate and severe/critical cases according to Wu et al. (Wu and McGoogan, 2020), and the outcomes were recorded. Mild disease was defined as the presence of clinical symptoms and no changes seen in computed tomography (CT) chest scans, and moderate cases included all those with respiratory symptoms associated with changes found in CT scans and oxygen saturation above 92%. Severe cases were defined by the presence of the following criteria: respiratory distress, with respiratory rate (RR) ≥ 30/min, resting blood oxygen saturation <93% or partial pressure of arterial blood oxygen (PaO2)/oxygen concentration (FiO2) ≤ 300 mmHg and chest radiography showing more than 50% lesion or progressive lesion within 24–48 h. Critically ill cases included all severe cases that deteriorated who possessed respiratory rate (RR) > 30, oxygen saturation <92% at room air, partial pressure of arterial blood oxygen (PaO2)/oxygen concentration (FiO2) < 200 mmHg despite oxygen therapy with a chest radiography showing more than 50% lesion or progressive lesion within 24–48 h. Routine laboratory investigations, including complete blood counts (CBC) and measurements of liver and kidney functions were ordered for all patients. Other tests were ordered according to the clinical condition of the patient.

2.4. RNA extraction

Peripheral blood samples were collected in tubes containing ethylene diamine tetra acetic acid (EDTA) under complete aseptic conditions. Erythrocytes were lysed using buffer supplied by QIAGEN and used according to the manufacturer’s instructions. Total RNA was extracted from the lymphocytic cell pellets using the total RNA purification kit (Direct-Zol RNA Kit, Zymo Research, Germany), as described in the manufacturer’s instructions.

2.5. cDNA synthesis and RT-PCR

Total RNA (200 ng) was used as a template for synthesis of cDNA using the RevertAid First Strand cDNA synthesis kit (ThermoFisher, UK), according to the manufacturer’s instructions. The expression levels of the five genes were assessed using quantitative RT-PCR (qRT-PCR), which was conducted according to manufacturer’s instructions using SYBR™ Green real-time PCR (qPCR) master mix (Applied Biosystems, USA). The previously designed reverse and forward primers flanking mRNA transcripts of FOXP3, IFN-γ, TNF, IL-6 and COX-2 were used (Alexandre-Ramos et al., 2018; Daneshmand et al., 2016; Fu et al., 1999; Madec et al., 2009; Mencarelli et al., 2009). β-actin was used as a reference gene and as a control in the relative quantification method. Data were analysed using relative quantification of the cycle threshold (CT), and results were expressed using the 2^ΔΔCT (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Statistical analysis was performed using Minitab 17.1.0.0 for Windows (Minitab Inc., 2013; Pennsylvania, USA). Continuous data were presented as means and standard deviations (SD), and categorical data as numbers and percentages (%). The normality of the data was examined using the Shapiro-Wilk test. Comparison between the two groups of continuous data was performed using the independent t-test or Mann Whitney test for parametric and non-parametric variables, respectively, while more than two categorical groups were statistically analysed using the chi square test. Factors influencing the gene expressions of IFN-γ, TNFα, FOXP3, IL-6 and COX-2 were assessed using general linear models (GLM) with stepwise backward elimination. The accuracy of gene expression for predicting COVID-19 infection was assessed with receiver operating curve (ROC) analysis, assuming that the area under the ROC = 0.9 was significant with margins of type I error = 0.05 and type II error = 0.1. The validity of FOXP3 gene expression for predicting mortality in COVID-19 patients was evaluated with ROC analysis, assuming that the area under the ROC = 0.8 was significant with margins of type I error = 0.05 and type II error = 0.1. Simple linear regression equations were estimated to predict FOXP3 expressions from oxygen saturation percentage (SO2%). All tests were two-sided; P was considered significant if < 0.05.

3. Results

3.1. Patients’ characteristics

Among the 111 laboratory-confirmed COVID-19 positive patients,
mild and moderate cases represented 55% (n: 61), while the severe/critical cases represented 45% (n: 50) (Table 1). The severe/critical group was significantly older, with a mean age of 54 years. About 96% (n: 48) of those in the severe/critical group were diabetic and hypertensive. Cough and dyspnea were the significant clinical signs in patients in the group with the severe cases, while fever, headache and bone pain were more common among the mild and moderate cases.

3.2. Blood pictures and chemistry

Severe cases showed significantly lower levels of haemoglobin (Hb), total leucocytic (TLC) and lymphocyte counts, while having higher neutrophil counts in comparison to the mild and moderate cases. The blood chemistry was significantly higher in the group with the severe/critical cases than in those with mild and moderate cases and showed significantly higher levels of alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatinine (P < 0.001) (Table 1).

3.3. Gene expressions of FOXP3, IFN-γ, TNF, IL-6 and COX-2

A significantly greater downregulation of mRNA expression of IFN-γ, TNFα and FOXP3 was detected in COVID-19 patients in comparison to the control group (P < 0.001) (Fig. 1). On the other hand, IL-6 and COX-2 also had a significantly upregulated (P < 0.001). FOXP3 was found to be significantly upregulated in patients suffering from severe disease (P < 0.001) (Fig. 1, Table 1).

The possible role of FOXP3, IFN-γ, TNF, IL-6 and COX-2 in predicting COVID-19 is illustrated in Fig. 2. IFN-γ gene expressions were significantly different in the patient group than in the healthy control group, with the area under the curve (AUC) at 1 (P < 0.001). Furthermore, at a cut-off point of 9.02, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were equal to 100%. FOXP3 expression was excellent in predicting the disease, where the AUC was 0.9 (P < 0.001). The best cut-off point value was 11.62. Accordingly, sensitivity, specificity, PPV and NPV were 87%, 88%, 61% and 97%, respectively (Table 2, Fig. 2).

TNFα also has a good power of prediction, since the area under the ROC curve (AUC) is 0.76 (P < 0.001). The best cut-off point with excellent (100%) specificity was 0.37 and the sensitivity, PPV and NPV were 56%, 100% and 91%, respectively. The ability of the IL-6 and COX-2 gene expressions for predicting COVID-19 infection was very good, since the AUCs were 76% and 81%, respectively. Also, at cut-off points of >11.26 IL-6 expression, the sensitivity, specificity, PPV and NPV were 79%, 91%, 62% and 93%, respectively, and at cut-off points of >4.27 COX-2 expression, the sensitivity, specificity, PPV and NPV were 70%, 84%, 50% and 93%, respectively (Table 2, Fig. 2).

3.4. Factors influencing the gene expressions of different biomarkers

While the correlations of the expression of IFN-γ were significant with age and respiratory rates, as well as the presence of fever (P = 0.03, 0.04 and 0.04, respectively), the correlation of the expression of TNFα was significant with platelet counts, total leucocyte counts and the presence of headache (P < 0.001, <0.001 and 0.05, respectively) (Table 3). There was a significant association between the expression of FOXP3 and SO2%, RR, Hbs and creatinine levels (P < 0.001, 0.02, 0.04 and <0.001, respectively). The IL-6 expression was significantly influenced by being female and also by the presence of headache (P = 0.03). Finally, COX-2 expression was affected by age and lymphocytic counts, the presence of fever and SO2% (P = 0.001, 0.01 and 0.02, 0.01)

Table 1
Patients’ clinical and laboratory characteristics.

| Factors                  | Mild/Moderate (n = 61) | Severe/critical (n = 50) | P       |
|--------------------------|------------------------|--------------------------|---------|
|                          | Mean/Median/N          | SD/IQR/%                 |         |
| Age                      | 45.39                  | 15.97                    | 53.06   | 16.36   | 0.01*   |
| Sex (Male)               | 38                     | 62.29                    | 27      | 54      | 0.49*   |
| Comorbidities            | 10                     | 16.39                    | 24      | 48      | <0.001* |
| Diabetes mellitus        | 18                     | 29.50                    | 24      | 48      | 0.07*   |
| Hypertension             | 10                     | 16.39                    | 46      | 92      | <0.001* |
| Clinical and lab data    | 10                     | 16.39                    | 46      | 92      | <0.001* |
| Cough                    | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Dyspnea                  | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Chest pain               | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Fever                    | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Headache                 | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Bone ache                | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Fatigue                  | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Vomiting                 | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Diarrhea                 | 1                      | 1.63                     | 26      | 52      | <0.001* |
| Loss of smell            | 0.96                   | 95.97                    | 84.5    | (70.75-90) | <0.001* |
| SO2%                     | 0.3                    | 5.39                     | 4.5     | 5.7     | <0.001* |
| Respiratory rate         | 21                     | 4.27                     | 31      | (20-23) | <0.001* |

S: Mann Whitney test, $S$: Independent t-test, #: Chi square test, P considered significant if < 0.05. Continues data represented as mean (SD) or median (IQR), and categorical data as number (%).
Fig. 1. IFNγ, TNFα, FOXP3, IL-6 and COX2 mRNA expression in control and COVID-19 patients. a) IFNγ, b) TNFα, c) FOXP3, d) IL-6 and e) COX2.

Fig. 2. ROC curve of the mRNA expression in COVID-19 infected patients. a) IFNγ, TNFα and FOXP3 mRNA expression in predicting COVID-19 disease, b) IL-6 and COX2 mRNA expression in predicting COVID-19 disease, c) FOXP3 mRNA expression in predicting mortality among COVID-19 patients.
survivors and patients who died

The data represented as median and inter quartile rang (IQR), $\Sigma$: Mann Whitney

Comparison of mRNA expression of the different biomarkers in dead and

Table 4

Deterioration of the condition is usually accompanied by decreased

lymphocyte counts and high levels of D-dimer, as well as extensions of

lymphocyte counts, especially in the severe cases that were also corre

lated to COX-2 expression and SO2%. Pronounced lymphopenia and high serum pro-inflammatory cytokines had previously been reported to

lated to mortality (P<0.001). FOXP3 expression was the only biomarker in which there was a significant association between upre-

gulation and mortality (P<0.001) (Table 4). Moreover, FOXP3 expression was found to be a good predictive biomarker for mortality among COVID-19 patients, where the AUC was 0.76 (P<0.001) (Fig. 2). The best cut-off point was >0.63, with sensitivity, specificity, PPV and NPV at 70%, 75%, 38% and 92%, respectively.

4. Discussion

In the current study, there was a significant decrease in the lymphocyte counts, especially in the severe cases that were also corre-

lated to COX-2 expression and SO2%. Pronounced lymphopenia and high serum pro-inflammatory cytokines had previously been reported to

be associated with COVID-19 (Chen et al., 2020; Tan et al., 2020). The presence of hypoxia in association with upregulation of FOXP3 and

the production of Tregs was previously reported (Ben-Shoshan et al.,

major threatening conditions associated with COVID-19 that result from the cytokine storm that may precede acute lung injury or ARDS (Gaillelili et al., 2020; Ragab et al., 2020; Shimizu, 2019). Replacement of the FABP4+ macrophages with the inflammatory FCN1+ macrophages was detected in the lungs of patients who were severely affected by SARS-CoV-2. However, clonal expansion of CD8+ T cells, especially those specific for conserved coronavirus epitopes cells, was also detected in mild or moderate disease (Liao et al., 2020; Mallajosyula et al., 2021), and the presence of a greater number of these cells was associated with improved survival in patients with hematologic cancer who had COVID-19 (Bange et al., 2021).

In our cohort, FOXP3 expression was found to be significantly downregulated in COVID-19 patients. This proves that some degree of loss of the suppressive and regulatory functions of Tregs is associated with COVID-19 infection. However, progression occurs only in a pro-

portion of patients who may have other risk factors. It is interesting to note that, similar to previous reports, older ages, males and the presence of comorbidities were associated with the development of severe forms of the disease (Conti and Younes, 2020; Harrison et al., 2020; Palai-

dimos et al., 2020; Tan et al., 2020).

In addition, FOXP3 expression was found to be a good predictive biomarker for mortality, since it was upregulated in severe cases and coincides with the development of severe hypoxia and the deaths of patients. In a recent study, the ratio of Tregs to all CD4+ lymphocytes was three times higher in patients who had acute respiratory distress syndrome (ARDS) and did not survive and twice as high in survivors, compared to the control group (Adamzik et al., 2013). Moreover, the ratio of T regulatory lymphocytes to all CD4+ lymphocytes in the bronchoalveolar lavage (BAL) was found to be an independent prognostic factor for 30-day survival in this study group (Adamzik et al., 2013). Another study showed that blood Tregs/CD4+ percentages were higher in patients who developed ARDS than in those who did not, and that a threshold of 10.4% for the blood Tregs/CD4+ percentages during the first week of ARDS was able to distinguish survivors from non-survivors (Halter et al., 2020). While FOXP3 was generally downregulated in COVID-19, it was upregulated in severe cases, with the development of severe hypoxia. This suggests that increased FOXP3 expression is a good predictor of severe COVID-19. However, the exact role of FOXP3 in the pathogenesis of COVID-19 remains to be determined.
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Epidemiological evidence confirms hypoxia as a key factor in the severity of COVID-19. Several studies have shown that the production of pro-inflammatory cytokines was increased in patients with COVID-19 (Stephen-Victor et al., 2020). Our study concluded that SARS-CoV-2 infection is associated with the downregulation of FOXP3, with the latter being the marker of T regulatory lymphocytes, and upregulation of the inflammatory genes IL-6 and COX-2. As the disease progresses, upregulation of FOXP3 follows, and this is associated with the severity of hypoxia and the impending death of patients infected with COVID-19. This upregulation could, then, be used as a biomarker for disease deterioration. Using anti-inflammatory drugs early in the course of the disease could be useful to limit disease progression. The kinetic curve for gene expression of immune and inflammatory markers is also recommended to gain a better understanding of the interaction of SARS-CoV-2 with the host immune system.

5. Conclusion

Here, we concluded that that SARS-CoV-2 infection is associated with the downregulation of FOXP3, with the latter being the marker of T regulatory lymphocytes, and upregulation of the inflammatory genes IL-6 and COX-2. As the disease progresses, upregulation of FOXP3 follows, and this is associated with the severity of hypoxia and the impending death of patients infected with COVID-19. This upregulation could, then, be used as a biomarker for disease deterioration. Using anti-inflammatory drugs early in the course of the disease could be useful to limit disease progression. The kinetic curve for gene expression of immune and inflammatory markers is also recommended to gain a better understanding of the interaction of SARS-CoV-2 with the host immune system.

5.1. Limitations of the study

Our study has two major limitations. First, collection of a single sample form each patient is considered as a limitation of this study. We recommend collection of serial samples from each patient at different stages of the disease to monitor the change in the expression of these markers along with the progress of the disease. Second, we collected two types of clinical data about the patients: the clinical condition of the patient at the time of samples collection, and whether the disease led to survival or mortality. We haven’t studied the evolution of clinical condition of the patients during the course of the disease.

CRediT authorship contribution statement

Ahmed S. Abdelhafiz: Conceptualization, methodology, and writing—original draft preparation. Mariam A. Fouda: Conceptualization, methodology, formal analysis, and laboratory analysis. Mohamed M. Sayed-Ahmed: Conceptualization, methodology, and laboratory analysis. Mahmoud M. Kamel: Conceptualization, supervision, and validation. Asmaa Ali: Data processing, statistics, and formal analysis. Merhan Fouda: Data curation, samples, data collection and curation. Mahmoud A. Khalil: Data curation, samples, data collection and curation. Ahmed S. Abdel-Moneim: Writing – review & editing.

Lamya M. Kamal: Data curation, samples, data collection and curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence
the work reported in this paper.

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