Altered Transcript Levels of Cytokines in COVID-19 Patients

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Abstract

The pandemic caused by severe acute respiratory syndrome coronavirus 2 and the related disorder i.e. “coronavirus disease 2019” (COVID-19) have encouraged researchers to unravel the molecular mechanism of disease severity. Several lines of evidence support the impact of "cytokine storm" in the pathogenesis of severe forms of the disorder. We aimed to assess the expression levels of nine cytokine coding in COVID-19 patients admitted in a hospital. Expression levels of *IFN-G*, *IL-2*, *IL-4*, *IL-6*, *IL-17*, *TGF-B*, *IL-8* and *IL-1B* were significantly higher in COVID-19 patients compared with healthy controls and in both female and male patients compared with sex-matched controls. However, expression of none of these cytokines was different between ICU-admitted patients and other patients except for *IL-6* whose expression was lower in the former group compared with the latter (ratio of means = 0.33, P value = 4.82E-02). Expression of *TNF-A* was not different between COVID-19 patients and healthy controls. Then, we assessed diagnostic power of cytokine coding genes in differentiating between COVID-19 patients and controls. The area under curve (AUC) values range from 0.94 for *IFN-G* to 1.0 for *IL-2* and *IL-1B*. After combining the transcript levels of all cytokines, AUC, sensitivity and specificity values reached 1.0, 1.0 and 0.99, respectively. For differentiation between ICU-admitted patients and other patients, *IL-4* with AUC value of 0.68, had the best diagnostic power among cytokine coding genes. Expression of none of cytokine coding genes was correlated with the assessed clinical/demographic data including age, gender, ICU admission, or CRP/ESR levels. Our study provides further evidence for contribution of “cytokine storm” in the pathobiology of moderate/severe forms of COVID-19.

Introduction

“Severe acute respiratory syndrome coronavirus 2” (SARS-CoV-2) has been shown to cause “coronavirus disease 2019” (COVID-19). This disorder has led to catastrophic events all over the world. Several lines of evidence support the role of "cytokine storm" in the pathogenesis of severe forms of the disorder (1, 2). This term is described as an induction of a cascade of auto-augmenting cytokine secretion resulting from uncontrolled host immune reaction to certain immune stimulators (1). A number of recent investigations have demonstrated elevated levels of pro-inflammatory cytokines in the critically ill COVID-19 patients compared with moderately ill ones (3, 4). Induction of immune responses during the course of COVID-19 has been shown to result in the over-production of enormous quantities of inflammatory proteins, eventually leading to organ damage (5). Up-regulation of pro-inflammatory cytokines such as IL-6, IL-1β, IL-18, and TNF-α in addition to their downstream molecules have been reported in these patients (6, 7). Moreover, dysregulation of a number of other inflammatory molecules such as IL-2, IL-7, IL-10 and IFN-γ have been associated with COVID-19 (8). Totally, many studies have shown the important impact of IL-6, IFN-γ and TNF-α in the pathobiology of this condition and related organ damage (5). Thus, identification of these inflammatory reactions has significance in the treatment of patients with COVID-19 infection (9). Based on the diversity in the regulatory mechanisms of immune reactions in different ethnic groups (10), evaluation of cytokine production in the context of COVID-19 infection in each population would help in the design of appropriated therapeutic options. Therefore, in the present investigation, we analyzed expression levels of some cytokines including those participating in adaptive immune responses (*IL-2* and *IL-4*) and pro-inflammatory cytokines (*IFN-G*, *IL-1*, *IL-6*, *IL-17* and *TNF-α*) in a population of Iranian patients with COVID-19 in comparison with sex-/ age-matched healthy controls to unravel the importance of cytokine dysregulation in the pathogenesis of this disorder.

Patients And Methods

Study participants

The present investigation was performed on patients admitted to Nikan Hospital, Tehran during March 2020 till April 2020. Patients with clinical symptoms of COVID-19 were further assessed through RT-PCR assay on the obtained nasopharyngeal samples. Only those with confirmed molecular diagnosis were enrolled in the study. Control samples were obtained from healthy individuals without no clinical symptom or exposure to the affected individuals. The study protocol was approved by ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1399.046). Informed consent was obtained from all patients. A complete paraclinical investigation and chest CT scan were done for all patients.

Expression assays

As the initial step, 3-5 mL of peripheral blood was gathered from all patients and controls. Then, total RNA was recovered from these specimens using the GeneAll RNA extraction Kit (Seoul, South Korea). Subsequently, total RNA was converted to cDNA using the OneStep RT-PCR Series Kit (BioFact™, Seoul, South Korea). Expression levels of cytokine coding genes were quantified in samples obtained from COVID-19 patienst and controls using the RealQ Plus 2x Master Mix (Amplicon, Denmark) using the transcript levels of *HPRT1* gene as normalizer. Table 1 displays the features of primers, probes and amplified segments.

Table 1
Information of primers, probes and amplicons.

Amplicon length	Primer/ probe	Gene
88	F: AGCCTAAGATGAGAGTTC	*HPRT1*
	R: CACAGAACTAGAACATTGATA	
	FAM -CATCTGGAGTCCTATTGACATCGC- TAMRA	
163	F: ATAGCCTGGACTTTCCTGTTGTC	*IL-1B*
	R: GTGAGTAGGAGAGGTGAGAGAGG	
	FAM- ACACCAATGCCCAACTGCCTGCCT- TAMRA	
109	F: GGGATCTGAAACAACATTCATGTG	*IL-2*
	R: AGTCAGTGTTGAGATGATGCTTTG	
	FAM -TGATGAGACAGCAACCA -TAMRA	
88	F: TGCTGCCTCCAAGAACACAAC	*IL-4*
	R:GTCCTTCTCATGGTGGCTGTAG	
	FAM- CCGGAGCACAGTCGCAGCCCT- TAMRA	
160	F: ATGCAATAACCACCCCTGACC	*IL-6*
	R: CCATGCTACATTTGCCGAAGAG	
	FAM- ACCACAAATGCCAGCCTGCTGACG- TAMRA.	
77	F:CGGAAGGAACCATCTCACTGTG	*IL-8*
	R:AGAAATCAGGAAGGCTGCCAAG	
	FAM- TGACTTCCAAGCTGGCCGTGGCTC- TAMRA	
176	F: CAGCAAGAGATCCTGGTCCTG	*IL-17*
	R: GGTCGGCTCTCCATAGTCTAAC	
	FAM-AGCCTCCACACTGCCCCAACTCCT-TAMRA	
96	F: GGCAAGGCTATGTGATTACAAGG	*IFN-G*
	R: CATCAAGTGAAATAAACACACAACCC	
	FAM- AGGGGCCAACTAGGCAGCCAACCT -TAMRA	
101	F: GCTCCACGGAGAAGAACTGC	*TGF-B*
	R: GTTGGCATGGTAGCCCTTGG	
	FAM- CCACTTCCAGCCGAGGTCCTTGCG -TAMRA	
97	F: TCCACCCATGTGCTCCTCAC	*TNF-A*
	R: TCTGGCAGGGGCTCTTGATG	
	FAM- CTACCGAGTCCGTGTCTACCA -TAMRA	

Statistical methods

Statistical comparisons were performed using R programming language. Transcript quantities of nine cytokine coding genes were measured from Ct values, considering HPRT1 as the reference gene. The following formula was used:

$$\frac{amp_g^{-CT_g}}{amp_{HPRT1}^{-CT_{HPRT1}}}$$

where g indicates any of cytokine coding genes. Then the calculated values were log2 transformed and used for succeeding analyses. Two comparisons were done and the significance of difference in mean values between two subgroups was computed using the t-test. Comparisons included: 1. Comparison

between expression levels of genes between total patients and healthy controls, and 2. between patients who required admission in intensive care unit (ICU) and who did not. Correlations between expression levels were appraised through the calculation of Spearman correlation coefficients.

ROC curves were plotted using Bayesian Generalized Linear Model, Generalized Linear Model, and Linear Discriminant Analysis with 10-fold cross validation. The linear Discriminant Analysis (LDA) provided the most efficient estimates and in the best setting, the AUC was 1. Youden's J statistic was employed to find the optimum threshold. DA was then chosen according to the former steps to investigate efficiency of each gene for separating groups. Spearman correlation coefficients were employed to assess the association between patients' information and transcript levels. To compute correlation between categorical and continuous variables point biserial correlation coefficient was used. For all statistical tests, the level of significance was set at P value < 0.05.

Results

A total of 91 COVID-19 patients (female/ male ratio: 38/ 53) and 96 healthy subjects (female/male ratio: 39/ 57) entered the study. The mean age (standard deviation) of the patients was 57.17 (16.90) years. Among the admitted patients, 37 patients (40.6%) were admitted in the intensive care unit (ICU). Table 2 shows the paraclinical parameters of the COVID-19 group.

Table 2
Paraclinical parameters of the COVID-19 group.

Parameters	Mean	Standard deviation
WBC (10^9/L)	8.119	8.482
RBC (10^{12}/L)	4.681	0.768
HB (g/dL)	12.70451	2.182114
HCT (%)	39.26703	6.595187
MCV (fl)	83.98593	5.702471
MCH (pg)	27.15484	2.330278
MCHC (g/dL)	32.34879	1.372015
PLT (10^9/L)	210.354	95.216
LYM (%)	21.043	11.323
NEUT (%)	69.098	13.087
ESR (mm/hr)	44.131	32.701
CRP (mg/dL)	73.256	69.540

Expression assays

Figure 1depicts the relative expression levels of cytokine coding genes in COVID-19 patients and healthy subjects.

Expression levels of *IFN-G*, *IL-2*, *IL-4*, *IL-6*, *IL-17*, *TGF-B*, *IL-8* and *IL-1B* were significantly higher in COVID-19 patients compared with controls and in both female and male patients compared with sex-matched controls. However, expression of none of these cytokines was different between ICU-admitted patients and other patients except for *IL-6* whose expression was lower in the former group compared with the latter (ratio of means=0.33, P value=4.82E-02). Expression of *TNF-A* was not different between COVID-19 patients and healthy subjects (Table 3).

Table 3
Details of expression assays of cytokine transcripts in study groups.

Number of Samples		SE	Ratios of means	P Value	95% CI		SE	Ratios of means	P Value	95% CI		SE	Ratios of means	P Value	95% CI	
		IFN-G					*IL-2*					*IL-4*				
Patients/controls																
Total	91/96	0.41	72.48	2.14E-33	5.36	6.99	0.52	4119.25	4.54E-51	10.99	13.03	0.50	980.03	3.76E-48	8.96	10.92
F	38/39	0.59	169.13	5.54E-20	6.22	8.58	0.81	5788.51	1.07E-23	10.89	14.11	0.69	1468.67	1.07E-23	9.14	11.90
M	53/57	0.56	40.04	1.49E-15	4.21	6.43	0.67	3277.83	2.87E-28	10.34	13.02	0.69	733.21	2.03E-25	8.15	10.89
ICU/NON_ICU																
Total	37/54	0.60	0.68	3.54E-01	-1.77	0.64	0.89	1.08	8.99E-01	-1.66	1.89	0.66	1.06	9.07E-01	-1.24	1.39
F	13/25	0.84	0.34	7.86E-02	-3.31	0.19	1.41	1.40	7.32E-01	-2.41	3.38	0.80	1.08	8.90E-01	-1.51	1.73
M	24/29	0.88	1.20	7.63E-01	-1.50	2.03	1.20	0.88	8.77E-01	-2.60	2.23	0.99	1.19	8.04E-01	-1.73	2.23
		IL-6					*IL-17*					*TGF-B*				
Patients/controls																
Total	91/96	0.50	362.95	7.28E-38	7.52	9.49	0.60	8175.67	2.15E-47	11.82	14.18	0.38	680.19	1.16E-47	8.65	10.16
F	38/39	0.93	1031.33	5.38E-16	8.16	11.86	0.80	11850.00	1.03E-26	11.94	15.13	0.53	1058.46	3.36E-25	8.99	11.11
M	53/57	0.52	174.75	2.98E-25	6.41	8.49	0.85	6341.07	1.02E-23	10.93	14.33	0.53	495.45	4.08E-25	7.89	10.01
ICU/NON_ICU																
Total	37/54	0.81	0.33	4.82E-02	-3.22	-0.01	1.00	0.37	1.56E-01	-3.40	0.55	0.70	0.76	5.83E-01	-1.78	1.01
F	13/25	1.59	0.16	1.04E-01	-5.97	0.59	1.28	0.26	1.41E-01	-4.59	0.69	1.10	0.79	7.56E-01	-2.64	1.95
M	24/29	0.86	0.61	4.01E-01	-2.44	0.99	1.50	0.46	4.64E-01	-4.14	1.91	0.98	0.86	8.24E-01	-2.19	1.75
		TNF-A					*IL-8*					*IL-1B*				
Patients/controls																
Total	91/96	0.39	337.83	1.23E-48	7.63	9.17	0.45	1066.47	2.36E-50	9.17	10.95	0.43	3091.82	1.40E-55	10.74	12.45
F	38/39	0.51	730.85	1.36E-29	8.50	10.53	0.62	3226.20	5.94E-30	10.42	12.89	0.65	6261.08	5.52E-28	11.32	13.91
M	53/57	0.56	196.39	3.96E-23	6.51	8.72	0.61	489.96	5.58E-24	7.71	10.16	0.58	1880.18	1.67E-29	9.73	12.02
ICU/NON_ICU																
Total	37/54	0.63	0.92	8.55E-01	-1.37	1.14	0.74	0.93	8.88E-01	-1.58	1.37	0.80	1.35	5.89E-01	-1.16	2.03
F	13/25	0.90	0.92	8.91E-01	-2.02	1.77	0.97	0.73	6.43E-01	-2.44	1.53	1.30	1.23	8.21E-01	-2.43	3.03
M	24/29	0.93	1.07	9.21E-01	-1.78	1.96	1.07	1.33	7.05E-01	-1.75	2.56	1.06	1.67	4.89E-01	-1.39	2.87

Then, we assessed diagnostic power of cytokine coding genes in diagnosing between COVID-19 patients and healthy controls. The area under curve (AUC) values range from 0.94 for *IFN-G* to 1.0 for *IL-2* and *IL-1B*. After combining the transcript levels of all cytokines, AUC, sensitivity and specificity values reached 1.0, 1.0 and 0.99, respectively. For differentiation between ICU-admitted patients and other patients, *IL-4* with AUC value of 0.68, had the best diagnostic power among cytokine coding genes (Table 4).

Parameters obtained from ROC cu

	IFN-G			IL-2			IL-4			IL-6		
Samples	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
Patients/ Controls												
Total 91/96	0.94	0.94	0.82	1.00	1.00	0.98	0.98	0.95	0.96	0.98	0.97	0.89
ICU/NON_ICU												
Total 37/54	0.54	0.50	0.63	0.62	0.53	0.67	0.68	0.58	0.69	0.61	0.65	0.62

Figures 2 and 3 show the ROC curves depicted by three machine learning models and the best fitted one, respectively.

Expression of none of cytokine coding genes was correlated with the assessed clinical/demographic data including age, gender, ICU admission, or CRP/ESR levels (Figure 2).

Lastly, we measured the correlation between the transcript levels of cytokine coding genes among ICU-admitted COVID-19 patients, Non-ICU-admitted COVID-19 patients and healthy controls (Figure 5). In the first group, the most significant correlation was found between *TNF-A* and *TGF-B* (r=0.91, P value= 9.37E-15). In non-ICU admitted patients and healthy controls, the most significant correlations were demonstrated between *TNF-A* and *IFN-G* (r=0.91, P value= 1.30E-21 and r=0.92, P value= 7.90E-41, respectively).

Discussion

We examined expression levels of nine cytokine-coding genes among ICU-admitted COVID-19 patients, non-ICU-admitted ones and healthy subjects. We detected over-expression of *IFN-G*, *IL-2*, *IL-4*, *IL-6*, *IL-17*, *TGF-B*, *IL-8* and *IL-1B* in COVID-19 patients compared with healthy subjects and in both female and male patients compared with sex-matched controls. However, expression of none of these cytokines was different between ICU-admitted patients and other patients except for *IL-6* whose expression was lower in the former group compared with the latter. Expression of *TNF-A* was not different between COVID-19 patients and healthy subjects.

Levels of several cytokines have been assessed in different subgroups of COVID-19 patients. For instance, Chen et al. have reported remarkable over-expression of IL-2R and IL-6 in the critically ill COVID-19 patients compared with severe and mild groups (11). However, we detected a trend toward under-expression of *IL-6* in the ICU-admitted patients compared with the other group of patients. This finding is not reliable as sex-based comparisons did not verify the difference in the expression of this cytokine between ICU-admitted and non-ICY admitted subjects. Moreover, this finding is in contrast with our previous study demonstrating higher median levels of IL-6 protein in the serum of ICU-admitted patients (12). However, it is worth mentioning that the current study varies with our previous study in the terms of applied technique and source of expression assessment. Anyway, we recommend assessment of expression of IL-6 in larger cohorts of Iranian patients to unravel the possible difference in its expression between Iranian patients and patients from other populations.

Chen et al. did not detect difference in the serum levels of TNF-α, IL-1 and IL-8 among the critical, severe and moderate groups COVID-19 patients (11). This finding is in accordance with our finding regarding similar levels of these cytokine between ICU-admitted and non-ICU-admitted COVID-19 patients.

The observed over-expression of *IFN-G* in COVID-19 patients is in line with the recently reported augmented nucleoprotein-induced IFN-γ release in these patients (13). Hu et al. have demonstrated lower probability of lung fibrosis at discharge in patients who had higher baseline levels of IFN-γ (14). Although we did not assess the presence of lung fibrosis in the admitted patients, we demonstrated similar levels of *IFN-G* between ICU-admitted and non-ICU-admitted patients. Over-expression of *IL-17* in COVID-19 patients has also been reported in other populations (15). In addition, MERS-CoV has been shown to stimulate expression of this cytokine in humans (16). Consistently, Th17 cells have been reported to participate in the cytokine storm stimulated by SARS-CoV-2 (17). Up-regulation of IL-17, IL-2 and IL-4 levels have also been reported in COVID-19 patients with lung lesions (15).

Similar levels of *TNF-A* between three study subgroups in the current investigation raises the possibility of ethnic-based differences in the immunological responses in the context of COVID-19 infection, since this cytokine has been repeatedly reported to be increased in these patients and has been suggested as a target of immune-modulatory options (18). SARS-CoV-2 has also been suggested to activate of IL-1β, which consecutively induces other pro-inflammatory cytokines, such as IL-6 and TNF-α (18). However, while we detected over-expression of *IL-1B* and *IL-6* in COVID-19 patients, we could not demonstrate any significant difference in the expression of *TNF-A*.

Then, we measured diagnostic power of cytokine transcripts in diagnosing COVID-19 patients from healthy controls. The AUC value was highest for *IL-2* and *IL-1B*. After combining the transcript levels of all cytokines, AUC, sensitivity and specificity values reached 1.0, 1.0 and 0.99, respectively. Therefore, cytokine levels can be used for distinguishing disease status. For differentiation between ICU-admitted patients and other patients, *IL-4* with AUC value of 0.68, had the best diagnostic power among cytokine coding genes. Therefore, these molecules cannot differentiate subgroups of COVID-19 patients. Besides, expression of none of cytokine coding genes was correlated with the assessed clinical/demographic data including age, gender, ICU admission, or CRP/ESR levels.

Lastly, we evaluated the correlation between the transcript levels of cytokine coding genes among three study subgroups. Patterns of correlation between expression levels of genes were more similar between non-ICU admitted patients and healthy controls, implying the altered regulatory mechanisms of

cytokines expression in severely affected patients.

In brief, we demonstrated altered levels of several cytokine coding genes in Iranian patients with COVID-19 infection. Our study provides further evidence for contribution of “cytokine storm” in the pathogenesis of moderate/severe forms of COVID-19.

Declarations

Ethics approval and consent to Participant

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1399.046). All methods were performed in accordance with the relevant guidelines and regulations.

Consent of publication

Not applicable

Availability of Data and Materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Competing Interest

The authors declare they have no conflict of interest

Funding

Not applicable

Authors’ contributions

SGF and MT wrote the manuscript and contributed in study design. MS, RJK and HRK collected the data and confirmed the patients diagnosis. AS, SHS and SAJ analyzed the data. All authors approved the manuscript.

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