












Investigation of the Toll-like receptors expression profile in peripheral blood mononuclear cells in COVID-19 patients in Iran

Alireza Tabibzadeh¹ , Mohammad Hadi Karbalaie Niya^{1,2} , Hossein Keyvani¹ , Sajad Karampoor² , Parastoo Yousefi¹ , Mohammad Hossein Razizadeh¹ , Abdulhussain Kadhimi Jawaziri¹ , Jahanbakhsh Khamseh³ , Maryam Esghaei^{1*} 

¹Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

²Gastrointestinal and Liver Diseases Research Center, Iran University of Medical Sciences, Tehran, Iran

³Department of Internal Medicine, Razan Hospital, Hamedan University of Medical Sciences, Hamedan, Iran

*Correspondence to

Maryam Esghaei, Email:
esghaei.m@iums.ac.ir
maryam.esghaei@gmail.com

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Abstract

Introduction: Coronavirus disease 2019 (COVID-19) spreads all around the world and leads to several new infection cases and mortality. A better understanding of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pathogenesis could lead to more efficient therapeutic approaches.

Objectives: The current study aimed to investigate the Toll-like receptors expression profile in peripheral blood mononuclear cells in COVID-19 patients in Iran.

Patients and Methods: In this cross-sectional study, we evaluated 32 COVID-19 patients. At the admission time based on the disease severity patients were divided into two groups of severe and mild COVID-19. A group of 16 normal people was evaluated as the healthy control. Blood samples were collected before any treatment from patients at the admission time. Peripheral blood mononuclear cell isolation performed on blood samples. RNA is extracted from human peripheral blood mononuclear cells and use for the cDNA synthesis. The expression level of TLR-3, 4, and 7 were evaluated by using semi-quantitative real-time polymerase chain reaction (PCR).

Results: The patient's mean age was 57.12±3.08 years and 15 (47%) were male. The background disorders represent two patients in the severe group with respiratory disorders, four patients with cardiovascular disease, and seven patients with diabetes. The TLRs expression levels represent a higher expression of TLR-4 in COVID-19 in comparison with controls for TLR-4 ($P=0.007$), TLR-3 and 7 also represent up-regulation however there were no statistically significant differences in the relative fold changes (RFCs) between groups for all other evaluated TLRs ($P>0.05$).

Conclusion: The study highlights the importance of the TLR-4 in COVID-19 patients in expression level. Further studies for a clear conclusion about TLRs expression levels are recommended.

Introduction

Coronavirus disease 2019 (COVID-19) spreads all around the world and leads to many new infections. The WHO (Worlds Health Organization) reports on 21 March 2022, represents more than 469 million infected cases and 6 million deaths (1). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a betacoronavirus and is classified into the sarbecoviruses genus (2). The COVID-19 leads to a wide range of the clinical presentations and disease outcome in different populations or background medical conditions (3). Despite the mass vaccination in some countries, the virus represents new variants and new cases of vaccine breakthrough (4). In recent years, a great amount of knowledge provided about the SARS-CoV-2 pathogenesis and evolution (5).

The signaling pathways especially the IFN

Key point

Current study highlighted the importance of the innate immunity responses in COVID-19. Furthermore, the evaluation represents Toll-like receptor 4 (TLR4) as critical element in COVID-19 patients.

(interferon) and regulation of the innate immune responses are shaped by pattern-recognition receptors (PRRs) in pathogen-associated molecular patterns (PAMPs) detection (6). PRRs are classified into five major groups including toll-like receptors (TLRs), a retinoic acid-inducible gene I (RIG-I)-like receptors, nucleotide oligomerization domain (NOD)-like receptors, and some others (7).

TLR-7 and TLR-8 sense the single-stranded RNA (ssRNA) in endosomes while other TLRs are generally recognized the

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double-stranded RNA (dsRNA) (8). TLR-3 is the first TLR described to sense the dsRNA. The TLR-3, 7, 8, and 9 are intracellular components (8). PAMPs sense by the TLRs leads to downstream cascades and production of nuclear factor kappa B (NF- κ B) and interferon regulatory factors. These elements trigger pro-inflammatory cytokines and IFN production (9). Since the IFN is one of the major parts of the anti-viral immune responses, there are too many different approaches for the SARS-CoV-2 IFN response evasion that are introduced or hypothesized (10).

Objectives

A better understanding of the SARS-CoV-2 pathogenesis could lead to more efficient therapeutic approaches. The current study aimed to investigate the TLRs expression profile in peripheral blood mononuclear cells in COVID-19 patients in Iran.

Patients and Methods

Patients, sample preparation and clinical laboratory data

Referred patients to Iran University of Medical Sciences affiliated hospitals were included based on the inclusion criteria for the current study. The inclusion criteria were patients with a confirmed COVID-19 by the real-time polymerase chain reaction (PCR) for SARS-CoV-2 and written consent form. Furthermore, medication history before admission was evaluated and the patients with a history of any particular medications for COVID-19 except paracetamol and non-steroidal anti-inflammatory drugs were excluded. Patients based on the disease severity were divided into two groups of severe and mild. Severe patients were patients with clinical manifestation of COVID-19 pneumonia (oxygen saturation <90%) who needs supplementary oxygen support. Mild patients were patients with clinical manifestation of the COVID-19 who did not need supplementary oxygen support (oxygen saturation \geq 90%) or ventilation at the admission time. A group of 16 normal people was evaluated as the healthy control.

After obtaining the written consent, 5 mL of blood was collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. The blood is used for the peripheral blood mononuclear cell (PBMC) isolation. The PBMC isolation was performed by Ficoll density gradient

centrifugation.

Meanwhile, C-reactive protein (CRP) (ENiSon, Tehran, Iran), lactate dehydrogenase (LDH), ALT (alanine aminotransferase), AST (aspartate aminotransferase), and FBS (fasting blood sugar) were evaluated by commercially available kits (Parsazmon, Iran).

Nucleotide acid extraction and cDNA synthesis

The total RNA was extracted from the PBMC samples by using the Favorgene total RNA mini kit (Favorgene, Taiwan). The extraction was conducted based on the manufactures manual and the extracted RNA evaluated by NanoDrop spectrophotometer. The RNA was used for cDNA synthesis. The cDNA synthesis performed by the Yekta Tajhiz cDNA synthesis kit (Yekta Tajhiz, Iran) based on the manufacturer's protocol by using oligo DT primers. Moreover, 1 μ g of the synthesized cDNA was used for the Semi-quantitative real-time PCR.

Semi quantitative real time PCR for TLRs expression profile

The semi-quantitative real-time PCR for TLR-3, 4, and 7 expression profiles was performed by the specific primers and SYBR green PCR master mix based on the Livak method (11). Primers sequence and references are listed in Table 1. The thermal program was 20 seconds at 95°C, 20 seconds in optimum annealing temperature, and 30 seconds at 72°C repeated in 45 cycles. For the thermal program, Rotor-Gene Q (QIAGEN) was used. Beta-actin was used as the internal control in the real-time PCR assay. The expression level was represented by the relative fold change (RCF) by using $2^{-\Delta\Delta CT}$.

Statistical analysis

The real-time PCR fold changes statistical evaluation was performed by the *t* test. Chi-square and Pearson's tests were applied or statistical assessment of other variables. Statistically, evaluations were assessed using SPSS version 22 and the *P*<0.05 was considered as statistically significant.

Results

Patient's demographical and laboratory status

The patient's mean age was 57.12 \pm 3.08 years and 17 (53%) were Female. Background diseases were includes two

Table 1. The list of the primers and the annealing temperature for current study

Gene	Primer	Annealing temperature	Reference
TLR-3	Forward	CCT GGT TTG TTA ATT GGA TTA ACG A	(12)
	Reverse	TGA GGT GGA GTG TTG CAA AGG	
TLR-4	Forward	CTG CAA TGG ATC AAG GAC CA	(12)
	Reverse	TTA TCT GAA GGT GTT GCA CAT TCC	
TLR-7	Forward	TTA CCT GGA TGG AAA CCA GCT ACT	(12)
	Reverse	TCA AGG CTG AGA AGC TGT AAG CTA	
β -acting	Forward	TGGTTACAGGAAGTCCCTTGCC	(13)
	Reverse	ATGCTATCACCTCCCTGTGTG	

TLR-3: Toll-like receptor 3, TLR-4: Toll-like receptor 4, TLR-7: Toll-like receptor 7.

patients in the severe group with respiratory diseases, four patients (two patients in each group) with cardiovascular disease, and seven patients with diabetes (four in severe and three in mild groups). Seven of the severe patients were expired during the hospitalization. The clinical laboratory parameters are summarized in Table 2.

TLRs expression profile

The TLRs expression levels represent a higher expression of TLR-4 in COVID-19 in comparison with controls ($P=0.007$). There were no statistically significant differences in the RFC between severe, mild, and control groups for TLR-3, 4, and 7 ($P > 0.05$; Table 3).

Discussion

COVID-19 pandemic impacts in different aspects of life in recent years (14). The pandemic condition is continued by the virus evolution and introducing new variants of concern (15). Despite the great amount of research in last years, there are some features of the disease pathogenesis is described (16). The current study aimed to investigate the TLRs expression profile in peripheral blood mononuclear cells in COVID-19 patients in Iran.

Table 2. Laboratory features of 16 sever and 16 mild COVID-19 patients

Laboratory variable	Sever/Mild	Mean \pm SD	P value
LDH	Severe	660.64 \pm 59.76	0.20
	Mild	548.06 \pm 59.19	
ALT	Severe	65.52 \pm 15.95	0.49
	Mild	52.33 \pm 9.50	
AST	Severe	75.29 \pm 10.03	0.20
	Mild	57.66 \pm 8.75	
FBS	Severe	179.93 \pm 19.80	0.64
	Mild	163.88 \pm 25.62	
CRP	Severe	74.70 \pm 12.34	0.39
	Mild	59.93 \pm 10.91	

LDH: Lactate dehydrogenase and the value was IU/L, ALT: Alanine Aminotransferase and the value was IU/L, AST: Aspartate aminotransferase and the value was IU/L, FBS: Fasting Blood Sugar and the value was mg/dL, CRP: C-Reactive Protein and the value was mg/L

It seems to be the TLRs can play different parts in COVID-19. The TLRs are the innate immune sensors and can induce sufficient inflammation to stop the virus spread and adaptive immune responses while they can lead to pro-inflammatory cytokine production (17). These pro-inflammatory cytokines and uncontrolled immune response in the form of the cytokine storm are considered as one of the major parts of COVID-19 pathogenesis (18).

In the current study, we evaluate 32 COVID-19 patients in two different groups based on the disease severity. TLRs expression levels represent a higher expression of TLR-4 in COVID-19 in comparison with controls ($P=0.007$). There was an up-regulation in the TLR-3 and TLR-7 more than 2 folds in COVID-19 patients in comparison with healthy controls. However, this up-regulation did not represent any statistical significance differences ($P > 0.05$). Conducted studies hypothesis the effect of the augmented TLR downstream signaling in obesity may be a clue for severe COVID-19 in patients with underlying obesity (19). Overexpression of the PRRs especially the TLR in COVID-19 non-survivor patients is represented previously (20). TLRs as the main element in hyper inflammation and disease severity were proposed in other studies (21). For example Aguida et al (22) represented that, using infrared therapy can stop the cytokine storm and decrease the TLR-4 in COVID-19 patients. These data highlighted the importance of the TLR-4 associated inflammation in COVID-19. All these studies confirm the current study result about the TLR overexpression in COVID-19 and highlight the hypothesis of TLR antagonists as a therapeutic agent for severe COVID-19 (23). The importance of TLR-4 in COVID-19 severity and cytokine storm also can be illustrated by association of the TLR-4 polymorphisms with the severe disease, while the TLR-3 and TLR-7 polymorphisms did not show any association with the disease severity (24). TLR-4 in COVID-19 also is an inflammatory activator of vascular endothelial cells in the angiotensin-converting enzyme 2 (ACE2) -independent pathway (25). To our knowledge, current study is the first study directly aimed to evaluate the expression level of these three TLRs in COVID-19 severe, mild, and control groups.

Table 3. Relative fold change (RFC) of TLRs in COVID-19 and controls

Group	TLR-4		TLR-3		TLR-7	
	RFC	P value	RFC	P value	RFC	P value
Control	1.18 \pm 0.27	-	1.00 \pm 0.05	-	1.02 \pm 0.11	-
Mild	17.78 \pm 8.13	0.06	2.61 \pm 0.86	0.19	5.10 \pm 3.36	0.16
Severe	18.13 \pm 8.29	0.06	4.13 \pm 2.24	0.09	2.73 \pm 1.14	0.25
COVID-19 patients*	17.95 \pm 5.81	0.007**	3.37 \pm 1.21	0.06	3.92 \pm 1.79	0.12

P values have been calculated by the two tailed t test and unequal variances for comparing between mild or severe groups with controls.

* COVID-19 patients represent the collection of both groups of mild and severe disease.

** Represents a statistically significant value

Conclusion

The study highlights the importance of the TLR-4 in COVID-19 patients in expression level. Further studies for a clear conclusion about TLRs expression level in COVID-19 severe or mild cases are recommended.

Limitations of the study

It needs to be noted that, this study is preliminary and has some limitations. A major limitation of the current study was limited sample size that might be the reason for non-significant statistical up-regulation of the TLR-3 and 7 between healthy and COVID-19 patients.

Authors' contribution

Conceptualization: AT, ME.

Methodology: SK, AKJ, JK.

Validation: SK, AKJ, JK, AT.

Formal analysis: SK, AKJ, JK.

Investigation: AT, ME.

Resources: SK, AKJ, JK.

Data curation: MHKN, AT, HK, PY, MHR

Writing—original draft: AT, ME, MHK, PY, MHR, and HK.

Writing—review and editing: All authors.

Visualization: AT, ME.

Supervision: AT, ME.

Project administration: ME.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of Iran University of Medical Sciences approved this study (IR.IUMS.FMD.REC.1400.187). Accordingly, written informed consent was taken from all participants before any intervention. This study was extracted from PhD thesis of Alireza Tabibzadeh at the department of medical virology of Iran University of Medical Sciences (Thesis No. 22 in medical School). Additionally, ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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