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Potential Role of *TLR3* and *RIG-I* Genes Expression in Surviving COVID-19 Patients with Different Severity of Infection

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Abstract

Immunological genes, including *TLR3* and *RIG-I*, have recently been established to have linked to predisposition to coronavirus disease 2019 (COVID-19) and its severity. The purpose of this case-control study (100 recovered COVID 19 cases and 100 healthy individuals) was to determine the role of gender, age, TLR3 and RIG-I genes in COVID-19 aggressiveness. *TLR3* and *RIG-I* gene expression was detected using a quantitative real-time polymerase chain reaction (qRT-PCR). COVID-19 infection intensity increased with age and no statistical difference between males and females ($p>0.05$) was found. *TLR3* and *RIG-I* gene expression levels were higher in patients compared to healthy which is positively connected to infection severity development. The aforementioned genes have a favorable relationship in screening COVID-19 infection. According to receiver operating characteristic curve these genes have high sensitivity in assessing COVID-19 infection. This study found that age, *TLR3* and *RIG-I* genes may play a role in COVID-19 predisposition worsening.

Keywords: COVID-19, Gene expression, PRRs, Immunological genes, *TLR3* and *RIG-I* genes.

الدور الفعال لتعبير جينات *TLR3* و *RIG-I* في مرضى كوفيد-19 المتعافين من اصابات مختلفة الشدة

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الخلاصة

ارتبطت قابلية الإصابة بمرض فيروس كورونا 2019 (كوفيد-19) وشدة المرض مؤخرًا بالجينات المناعية مثل جينات *TLR3* و *RIG-I*. هدفت دراسة المرضى والاصحاء هذه (100 مريض متعافي من كوفيد-19 و 100 اصحاء) إلى تقييم أهمية الجنس ، العمر، وجينات *TLR3* و *RIG-I* في شدة مرض كوفيد-19. يتم قياس التعبير الجيني لجينات *TLR3* و *RIG-I* عن طريق تقنية تفاعل البلمرة المتسلسل الكمي بالزمن الحقيقي (qRT-PCR). أظهرت النتائج عدم وجود فروق احصائية بين ذكور واناث مرضى COVID-19 ، وظهر أن شدة الإصابة تزداد مع تقدم العمر. كما بينت النتائج زيادة مستويات التعبير الجيني لجينات *TLR3* و *RIG-I*في المرضى مقارنة بالاصحاء ، وهذه الزيادة مرتبطة بشكل إيجابي بتطور شدة

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العدوى. هناك ارتباط إيجابي بين جينات *TLR3* و *RIG-I*. كشف اختبار منحنى تشغيل المستقبل ROC عن حساسية عالية لجينات *TLR3* و *RIG-I*في توضيح عدوى كوفيد-19. في الختام ، أشارت الدراسة إلى الدور المحتمل الى العمر، بالإضافة الى جينات *TLR3* و *RIG-I* في حساسية التعرض لشدة كوفيد-19.

1. Introduction

The clinical manifestations of SARS-CoV-2 illness are extensive, spanning from silent or mild variants to acute severe pneumonia with serious lung damage and mortality [1]. SARS-CoV-2 intensity and death have been linked to old age, overweight, chronic illnesses and vitamin D deficiency [2]. Furthermore, the intensity of COVID-19 is compounded by an enhanced inflammatory response generated by SARS-CoV-2 which manifests itself as increased levels of inflammatory indicators, mainly pro-inflammatory mediators [3].

The immune response includes two types of reactions: adaptive and innate [4]. Adaptive immunity detects pathogens-associated molecular patterns (PAMPs) which are defined by pattern recognition receptors (PRRs)which are mostly represented in innate defense cells [3]. Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat carrying receptors (NLRs) and retinoic acid-inducible gene-I (RIG-)-like receptors are some of the PRRs that have been identified (RLRs) [4]. TLRs may play a role in both the initial rejection of antiviral therapy as well as the later growth of the catastrophic clinical symptoms of extreme COVID-19, basically ARDS with terminal respiratory arrest [5]. Interestingly, whereas TLR3 is favorable in the early stages of antiviral therapy high expression can lead to hyper inflammation and cytokine storms which are common in serious COVID-19 infections [5]. The good binding selectivity of SARS-CoV non-structural proteins 10 (NSP10) with TLR3 leads to its downstream induction [6].

While RLRs could be captured by SARS-CoV proteins to avoid the host immune system [7], very little is understood about their differential involvement in SARS-CoV-2 illness. The removal of RIG-I (retinoic acid-inducible gene I) seemed to have no effect on the generation of interferon (IFN) reactions by SARS-CoV-2, but somehow it does increase viral replication, indicating that RIG-I has an antiviral function that is independent of IFN [8]. RIG-I production was scarcely detectable in human bronchial epithelial cells obtained from two independent individuals with chronic obstructive pulmonary disease (COPD) according to Yamada *et al.* [9]. The anti-SARS-CoV-2 effect of melanoma differentiation associated protein 5 (MDA5) was found to be crucial but RIG-I-role in restricting SARS-CoV-2 infection was found to be unnecessary [10]. Thus, the conducted study aimed to assess the significance of gender, age and gene expression of *TLR3* and *RIG-I* in the severity of COVID-19 in Iraqi individuals with COVID-19.

2. Materials and Methods

2.1 Populations Studied

A case-control study was conducted from February April 2021 to evaluate the levels of *TLR3* and *RIG-I* genes expression in the Iraqi COVID-19 recovered individuals. Consecutive 100 cases (53 males and 47 females) who had recovered from COVID-19 were studied from the health centers and hospitals within Diyala province. The WHO 2020 guidelines on COVID-19 infection divided the cases into mild to moderate, severe and critical, depending on the specialist or physician's diagnosis and recovery time. These cases were distributed into 14 mild to moderate, 48 severe and 38 critical. The 100 healthy individuals samples included 67 males and 33 females. Their asymptomatic status, COVID-19 antibodies and the reverse transcriptase real-time PCR (RT-PCR) results were negative. The age means ± standard deviation of patients and healthy was 32.63 ± 12.10 and 30.83 ± 16.49 years accordingly.

2.2 Quantitative Gene Expression of TLR3 and RIG-I Genes

2.2.1 RNA Extraction from Blood Samples

Fresh venous blood samples (3 ml) were collected from all 100 COVID-19 recovered patients after being diagnosed with the infection through examination in the RT-PCR in the Public Health Department, Baquba Teaching Hospital, Diyala Health Department. Blood samples were similarly collected from 100 healthy individuals. The blood samples collected in EDTA tubes were then immediately transferred in a tube containing 600 µl of Tizol and mixed well. The tubes were then stored at -20 °C until use for RNA extraction to study the gene expression of TLR-3 and RIG-I genes. The RNA extraction from blood samples of both the recovered COVID-19 and healthy cases was achieved using TransZOl Up plus RNA kit (Transgen, China) and according to the manufacturer's instructions.

2.2.2 Two-step Quantitative Real Time-PCR for Gene Expression

A- gDNA Elimination and cDNA Production

The complementary DNA (cDNA) production was performed by utilizing protocol within EasyScript® One-Step gDNA deleted and complementary DNA generation Super-Mix (Transgen, China).

Procedure

3-5 µL of extracted RNA was transferred to RT-PCR tubes containing 1 µL Anchored Oligo (dT) Primer (0.5µg/µl) with 1 µL Random Primer (0.1µg/µl) and then incubated in thermocycler at 65°C for five minutes and 4°C for ten minutes). After incubation, it was added to 10 µL EX reaction mix; 1 µL gDNA remover, 1 µL Easy Script®RT/RI enzyme mix and 3 µL RNase-free water to complete a final volume of 20 µL.

Incubation in thermocycler is shown below:

A- Step1: temperature (25°Cc), time (10min) and purpose (Random Primer (N9) (primer binding)).

B- Step2: temperature (42°Cc), time (15min) and purpose (Anchored Oligo (dT)18 (extension)).

C- Step3: temperature (85°Cc), time (5seconds) and purpose (Inactivate reverse transcriptase enzyme (inactivation)).

B- Amplification of cDNA

The amplification on cDNA was done using TransStart® Green qPCRSuper mix kit with specific primers (Transgen, China). This process included several primers: *TLR-3 gene* (Forward-TCTGGAAAGGCGCAACC and Reverse-CCGTTGGACTCTAATTCAAGAT) (Medhi *et al.* 2011), *RIG-I gene* (Forward-TGATGAATGCCACAACACTAGT and Reverse-GGCCTGAAGATCCTCCAAGT) (designed in this study), and *Beta-actin* (*Housekeeping genes*) (Forward-GAAGGATTCCTATGTGGGCG and Reverse-TGGTGGTAAAGCTGTAC) (Medhi *et al.* 2011).

The following steps were followed to carry out the amplification in a reaction volume of 20 µl:

1 - Sybr Green qPCR Super Mix were defrosted at ambient temperature (25°C).

2 - Component's volume required to prepare the required number of reactions, were calculated as below:

Master mix SybrGreen (10 µl), forward primer (1 µl), reverse primer (1 µl), cDNA (3 µl), nuclease free water (N.F.W) (5 µl).

3 - All components were placed in a tube that was quickly spun to tumble down the material and to remove any unwanted bubbles.

4 - qRT-PCR program conditions included several stages as following:

A- Stage 1: initial denaturation included: 94ºC temperature for 30 minutes, 1 cycle.

B- Stage 2: denaturation 94ºC, annealing 58ºC and extension 72ºC, time (5 , 15, 20 seconds respectively), 40 cycles.

C- Stage 3: dissociation included: temperature 55-95ºC temperature, 1 minute time and 1 cycle.

2.3 Statistical Analysis

The quantities of RIG-I and TLR3 genes expression had first been checked for normalcy using Kolmogorov-Smirnov and Shapiro-Wilk test kits. Parameters having fit these tests (no large differences) were officially named Mean ± SD. Error and significant differences between the means were calculated using the Student t-test for comparing two numerical variables and the F test for comparing three or more numerical variables. The parameters that won't pass the normality tests (large differences), were reported as median and range, with Mann-Whitney tests used to determine whether there was a notable change between them (for comparison between two groups). The other factors were expressed as percent frequencies. Pearson-Chi-square tests were used to see whether there were significant differences in frequency. The kind and degree of the association between variables were explained using Pearson correlation (R). A receiver operator characteristic (ROC) curve was created for every variable and the area under the curve (AUC), sensitivity and specificity were calculated as a result. A p-value of less than 0.05 was deemed significant. These analysis were carried out using the statistical tool SPSS version 25.0 and Graph pad prism V.6.

3. Results and Discussion

3.1 Baseline Characters of Participants

Regarding the age group, our study discovered that there were significant differences (*p<0.05*) among age groups for patients and healthy individuals, where the age group 19-40 years scored highest percentage for patients and healthy,68% vs 45%. The age group >60 and ≤18 years scored least percentage for patients, 2% and 7% respectively. While 41-50 and 51-60 age groups scored least percentage for healthy, 8% and 7% respectively with significant difference (*p<0.05*) (Table 1).

Table 1 - Comparative anthropometric characters of the studied groups using Chi-square test.

Anthropometric characters			Groups		Total	*P* value
			Patients (n=100)	Healthy (n=100)		
Gender	Male	n	53	66	119	*P>0.05*
		%	53.00%	66.00%	59.50%	
	Female	n	47	34	81	*P>0.05*
		%	47.00%	34.00%	40.50%	
	P value		*P>0.05*	*P<0.01***	*P<0.01***	
Age groups (years)	≤18	n	7	30	37	*P<0.001****
		%	7.00%	30.00%	18.50%	
	19-40	n	68	45	113	*P<0.05**
		%	68.00%	45.00%	56.50%	
	41-50	n	13	8	21	*P>0.05*
		%	13.00%	8.00%	10.50%	
	51-60	n	10	7	17	*P>0.05*
		%	10.00%	7.00%	8.50%	
	>60	n	2	10	12	*P<0.05**
		%	2.00%	10.00%	6.00%	
	P value		*P<0.001****	*P<0.001****	*P<0.001****	
Mean±SD			32.63±12.16	26.53±4.51	31.83±14.51	*P<0.05**

The current findings demonstrated that 53% of males had a greater rate of COVID-19 illness than females which was 47%). Hence, no significant differences were recorded between them (P>0.05). Wolf *et al.* [2] noticed that the males comprise 54.3–57.3% of hospital admissions and 61.1% of intensive care unit (ICU) patients in China [2].However our study revealed that the male patients comprised 41-46% of hospital admissions and 55.62% of intensive care unit (ICU) in Baghdad. According to Krieger *et al.* [11] males accounted for 62% of in-hospital mortality in Wuhan.

Male's number instances were identical to the female's number instances. However, men had nearly twice the chance of dying from COVID-19, guiding to a variety of ideas around behaviors, hormones (e.g., Estrogen), the pathophysiology of each organ system and chromosomal structural differences [11]. According to a recent survey, females' innate immunological defenses are much stronger than males [12]. Nonetheless, males have higher levels of pro-inflammatory cytokines and chemokines according to the study applied by Draif *et al.* [3]. Also the fundamental cytokines storm, IL-6 receptor, is significantly abundant within lung epithelial cells in males, indicating that males are much more sensitive to it, which could contribute to COVID-19 worsening [12]. These observations were in accordance with our results that showed high frequency of COVID-19 in males.

The current study found significant differences ($p<0.05$) among age groups for COVID-19 patients and healthy individuals in 19-40 year olds comprising the greatest percentage, 68 % and 45 % accordingly, than other groups. These age discrepancies between patients and healthy people, as well as among patients, suggested that age is a potential risk factor, indicating that older people are more likely to become infected with COVID-19. This conclusion appeared to be consistent with a recent research that showed the relationship between the age and COVID-19 disease [13]. Lower angiotensin-converting enzyme 2 (ACE-2) production throughout the nasal epithelium of kids may well be the cause of reduced SARS-CoV-2 sensitivity, and hence minimal or non-existent COVID-19 disease in infants [14]. This can explain the lower percentage rates of the positive COVID-19 infections among younger age groups. COVID-19 fatality is more age-dependent than in fatalities from other diseases. Males have an increased risk in comparison to the females, which is less obvious as they get older [15]. Over 65 years individuals have a potential risk of COVID-19-related incidence and death than other age groups, making them to be prioritized for COVID-19 immunization [16]. COVID-19 infection is more common in patients with age progression which could be due to a lowered immune system, chronic illnesses, malnutrition, increased ACE-2 expression and/or organ failure [17].

3.2 Mean Levels of RIG-I and TLR3 Genes Levels within Study Groups

Results of the current study reported high median levels of RIG-*I* and *TLR3* genes in patients than healthy cases, 3.378; 0.686 and 2.394; 0.629 respectively. It appeared that there were significant differences between for *RIG-I* and *TLR3* genes (*p<0.05*) (Table 2).

Table 2 - Comparison of gene expression of *RIG-I* and *TLR3* genes between study groups by Mann-Whitney test.

Groups		N	Median	Minimum	Maximum	Range	Statistics
RIG-I gene	Patients	100	3.378	0.338	14.440	14.102	Mann-Whitney U= 673.00 *P<0.001***
	Healthy	100	0.686	0.211	1.777	1.566	
TLR3 gene	Patients	100	2.394	0.449	9.607	9.158	Mann-Whitney U= 1693.00 *P<0.001***
	Healthy	100	0.629	0.041	1.258	1.217	

The current investigation found that COVID-19 patients have higher TLR3 gene expression levels than that of healthy people, which agreed with results recorded by other investigators [18]. Toll-like receptors (TLRs) are the most well-known because of the host defense system's

PRRs and play an important role in recognition and fighting against pathogens, including Coronaviruses (SARS Co-V2) [6]. TLRs7/8 identify single-stranded positive-sense RNA, while TLR3 is specified for the double-stranded RNA intermediately generated throughout viral replication [19]. TLR3 stimulation is useful in the treatment of a variety of RNA virus infections [20]. TLR3 has been implicated in the formation of a protective response towards coronaviruses in several investigations on SARS-CoV and MERS [21]. Though TLR3 is helpful in the early stages of viral clearance, hyperactivation can lead to hyperinflammation and cytokine storms, which are common in extreme forms of the disease [5]. The good binding selectivity of SARS-Cov non-structural proteins 10 (NSP10) with TLR3 leads to induction of TLR3 downstream [6]. TLR3 has also been shown to play a protective function in COVID-19 infection outbreaks, including SARS-CoV1 and the Middle East respiratory sickness (MERS-CoV) in earlier investigations [22]. Some investigators found that TLR3 transcription increases after coronavirus illness as early as the second day following infection [21]. This can help determine the stimulation of downstream molecules like TIR-domain-containing adapter-inducing interferon (TRIF) which also helps determine the stimulation of transcriptional regulators like interferon regulatory factor-3 (IRF3) and nuclear factor-kappaB (NF-kB) that stimulate large production of type I interferons (IFN alpha and beta), inflammatory mediators (IL-6, TNF) and IFN-gamma, where these results were compatible with our results showing high levels of TLR3 in coronavirus infections in patients than the healthy. Researchers have investigated whether a mixture of toll-like receptors (TLR)1/2 and TLR3 contrast (L-pampo) can become a powerful adjuvant for SARS-CoV-2 subunit vaccine and have discovered that TLR agonists, L-pampo, can become a potent subunits vaccine to encourage adequate resistance mechanisms (cellular and humeral) against SARS-CoV-2 [23]. These results helped us in using TLR3 in therapy and vaccination against for SARS-CoV-2.

In contrary to viral RNA-detected TLRs (TLR3, TLR7) which are primarily found in the protective cells, Rig-like receptors (RLRs) signaling is active within tissues and cell kinds and consists of MDA5 and RIG-I proteins [24]. MDA5 loss had a comparable effect on viral replication and type I/III IFNs as mitochondrial antiviral signaling protein (MAVS deletion did [8], indicating that MDA5 is the main protein of RLR. RIG-I removal did not affect SARS-CoV-2 activation of IFN reactions. However, it did raise viral replication, thus indicating that RIG-I has an antiviral role that is distinct from MAVS-IFN. Even 1 hour after infection, RIG-I/ cells had a 2.5-fold increase in ACE2 expression relative to the wild type and other knockout cells. This showed that RIG-I might prevent SARS-CoV-2 from infected cells by suppressing ACE2 messenger RNA expression [25]. These results show importance of RIG-I in resistance toward SARS-CoV-2 . RIG-I could stimulate antiviral effector expression through a method that is not dependent on the IFN-Janus kinase (JAK) signal transducer and activator of transcription (STAT) [25]. RIG-I inhibited viral replication by disrupting the engagement of hepatitis B virus polymerase with the 5'-region of viral pre-genomic RNA in an RNA-binding dependent way [26]. MDA5 has a significant anti-SARS-CoV-2 activity, although RIG-I has a little involvement in suppressing SARS-CoV-2 infectious, according to a study [10]. In a nutshell, researchers discovered that MDA5 has a prominent role in recognizing SARS-CoV-2 and triggering immunological responses and that RIG-I has an IFN-independently anti-SARS-CoV-2 effect. According to the authors, RNA sensing of SARS-CoV-2 in lung epithelium is a primary cause of inflammation, the degree of which is regulated by the inflammatory process of the local environment, and targeting host defense responses may help decrease inflammation-related COVID-19 [27]. RIG-I expression amounts are significantly suppressed within most lungs cells isolated from sick individuals with chronic obstructive pulmonary disease (COPD) [28]. These results were not compatible to our results that showed high levels of RIG-I in patients with respiratory diseases. The

differences in RIG-I expressions are related to host immune status, pathogen type and infection site.

Researchers have discovered that when lung cells obtained from COPD patients were treated with All-trans retinoic acids (ATRA), the protein content of RIG-I was dramatically overexpressed in a dose-dependent way, whereas ATRA did not affect the amino acids manifestations of ACE2 and TMPRSS2 [9]. However, therapies with ATRA significantly reduced SARS-CoV-2 viral loads in such cells which reduced SARS-CoV-2 viral loads in these cells, which was reliant on RIG-I [9]. These findings imply that the RIG-I protein level is a key factor in anti-SARS-CoV-2 natural defense modulation. SARS-CoV-2 has a single-stranded positive-sense RNA that should be converted into a negative-sense RNA by viral RNA-dependent RNA polymerase (RdRp) in the early phase of viral multiplication, and as a result, the association of RIG-I with SARS-CoV-2 may impede this RdRp-dependent mechanism, according to the researchers [29]. Just the lack of RIG-I was positive-sense viral RNA collected with RdRp which did not affect RdRp protein expression levels. In a dose-dependent way, recombinant RIG-I (rRIG-I) but not recombinant MDA5 competitively blocked RdRp attachment to viral RNA [29]. The next step was to figure out which region(s) of the SARS-CoV-2 RNA genome is involved in the RIG-I connection. The RdRp has been shown to induce negative-strand manufacturing in those other SARS-related beta coronaviruses by gaining access to the 3′-untranslated region (3′ UTR) of the genome, which is consistent in terms of RNA secondary structure and sequence in SARS-CoV-2 [30]. Finally, high doses of RIG-I to patients with SARS-CoV-2 increases resistance toward this virus through prevent connecting RdRp to viral RNA. Therefore, we notice high levels of RIG-I in most recovered COVID-19. RIG-I level was scarcely detectable in human primary bronchial epithelial cells obtained from two independent COPD patients, according to Yamada et al. [9].

3.3 Mean Levels of RIG-I and TLR3 Genes Levels with COVID-19 Patients Infection Types

In relation to infection types, no significant differences were observed between mean levels of infection types in relation to RIG-I and TLR-3 genes.

TLR3 and RIG-I gene concentrations appeared to be increased with the intensity of COVID-19 illness, indicating an increase in the inflammatory process as the disease progressed. Although a well-regulated innate immunity is the primary line of defense against viral infections [31], serious COVID-19 infection causes hyper inflammation ("cytokine storm"), which can lead to acute respiratory distress syndrome (ARDS) [32]. TLR activation stimulates the nuclear factor-B (NF-B) sending signals ripple, having caused monocytes to produce markers of inflammation (interleukin -IL-1, tumor necrosis factor-alpha (TNF-α), and IL-6) in response to virus infections via direct anti-viral passageways, as well as the recruiting efforts of many other white blood cells [33]. Furthermore, the intensity of COVID-19 is influenced by the worsened oxidative stress generated by increased cytokine amounts, as well as lowered concentration levels of interferon ((IFN-α, IFN-β) [34]. According to other researcher findings, the SARS-CoV-2 N protein inhibits the IFN reaction by affecting the first stage in the host defense system, possibly the cellular PRR–RNA-recognition phase. As a result scientists believe that the SARS-CoV-2 N protein inhibits IFN-production by interacting with RIG-I [35]. SARS CoV-1 N protein, notably, can block IFN generation by inhibiting ubiquitination and stimulation of RIG-I [7]. By inhibiting the activation of NFB, the SARS CoV-1 M protein can also limit IFN signaling [36]. In general, the intensity of infection is based on immune status and viral dose. Therefore, we noticed increased levels of TLR3 and RIG-1 with the severity of COVID-19 infections and that because SARS-CoV-2 is firstly connecting with these proteins and then inhibits IFN production.

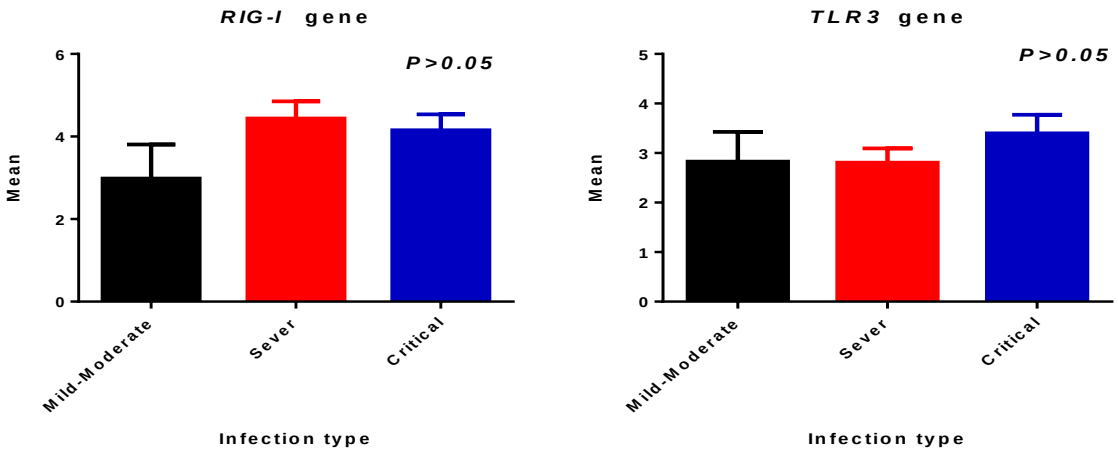

Figure 1-*RIG* and *TLR3* gene expression depending on the patient's infection types.

3.4 Correlation Relationship between RIG-I and TLR3 Genes

The current results showed a positive significant correlation between *RIG-I* and *TLR3* genes (r= 0.410** *p<0.01*) in COVID-19 patients (Figure 2).

The RIG-I and TLR3 genes were found to have a positive significant connection according to the current obtained results. Because the TLR3 and RIG-I sensors connect to viral double-stranded RNA molecules, this association boosts RIG-I and TLR3 gene production, which in turn boosts the inflammatory reaction by activating cytokines [37].

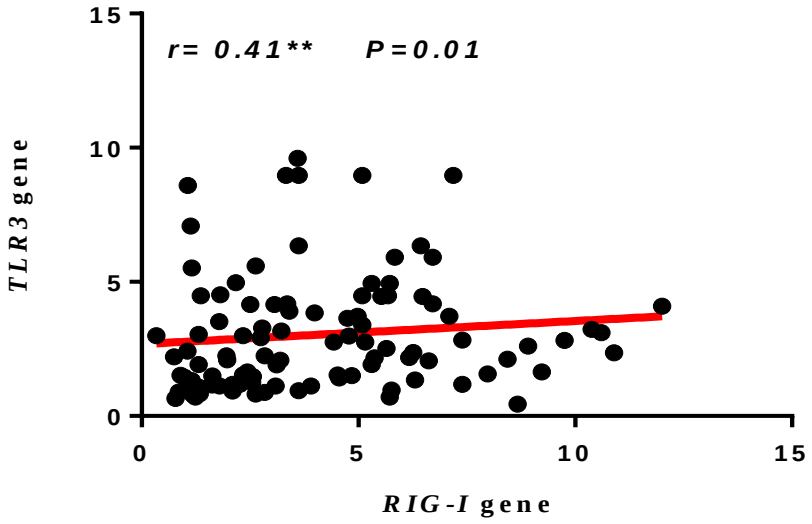

Figure 2-Relations between *RIG-I* and *TLR3* genes among COVID-19 patients

3.5 Receiver Operating Characteristic (ROC) Curve of RIG-I and TLR3 Genes

According to the receiver operator characteristic (ROC) curve, the current observations showed high sensitivity of *RIG-I* gene (AUC= 0.93 and Sn=95%) and *TLR3* gene (AUC= 0.83 and Sn=84%) in screening COVID-19 patients with significant differences (*P<0.05*). Based on specificity, *RIG-I* and *TLR3* genes scored low specificity, 29-% and 32% respectively (Figure 3).

Owing to such genes that encode TLR3 and RIG-1 receptors which are considered primary defensive lines of the protective immune response towards RNA viruses including coronavirus, the reactivity of RIG-I and TLR3 genes enhanced in evaluating patients with COVID-19 illness [38].

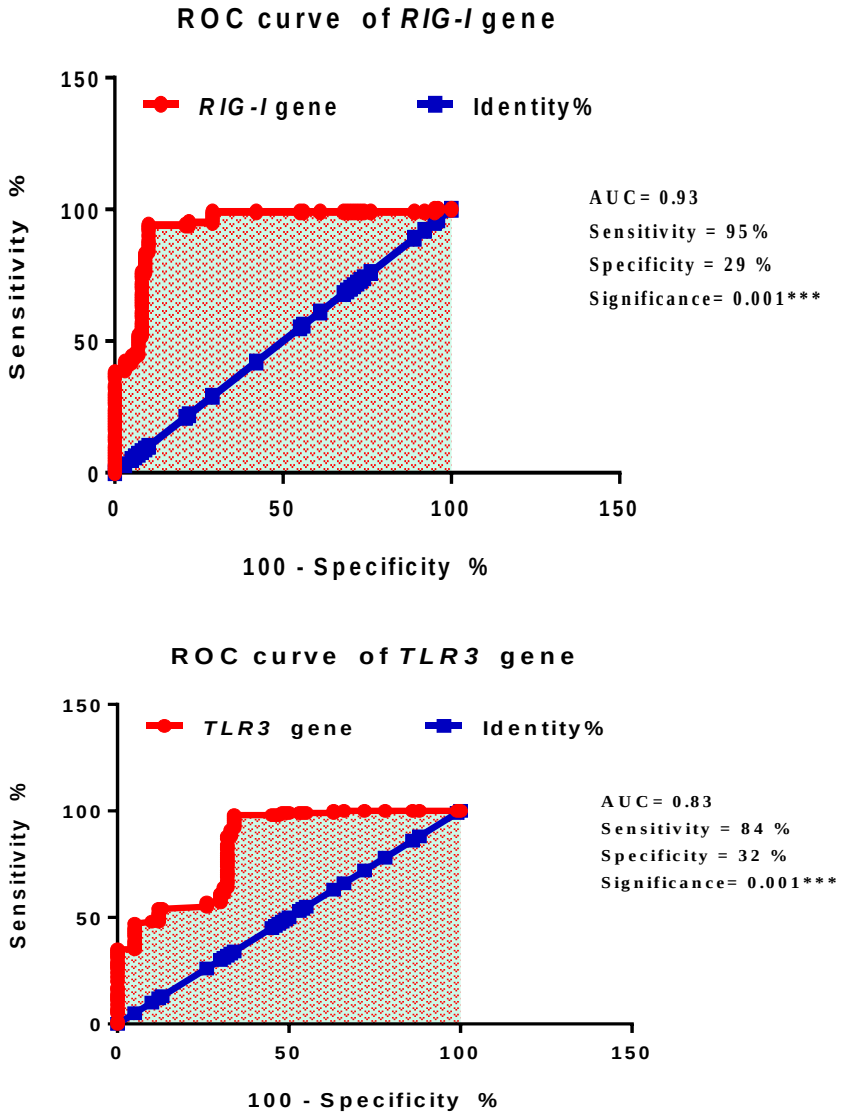

Figure 3- ROC curve of *RIG-I* and *TLR3* genes.

Study Limitations

The study shortcomings included the small number of patients and healthy individuals, particularly those with mild to moderate COVID-19 illness, and the necessity to distinguish between them.

4. Conclusions

In conclusion, this study indicated the major roles of the age, *RIG-I* and *TLR3* genes in susceptibility to COVID-19 severity. There is a positive significant correlation between *RIG-I* and *TLR3* genes.

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Conflict of Interests

There is no clear conflict, according to the authors.

References

[1] D. Mohammed, H. Tawfeeq, K. Ali, and et al., "Analysis and Prediction of COVID-19 Outbreak by the Numerical Modelling".*Iraqi Journal of Science*.62 (5),1452-1459, 2020.

[2] Z. Mahmood, H. Fadhil, A. Ad., ″Estimation of Hematological Parameters of Disease Severity in Iraqi Patients with COVID-19″. *Iraqi Journal of Science*, 3487-3496, 2021.

[3] D. Draif I. Hammi, A. Kihel and et al., ″The pro-inflammatory cytokines in COVID-19 pathogenesis: What goes wrong? ″. Microbial Pathogenesis, 104799, 2021.

[4] L. Koenderman, W. Buurman and M. Daha, ″The innate immune response″. *Immunology letters*, 162(2), 95-102, 2014.

[5] L. Onofrio, M. Caraglia, G. Facchini et al., ″Toll-like receptors and COVID-19: a two-faced story with an exciting ending″, 2020.

[6] A. Choudhury, N. Das, R. Patra, and et al., ″In silico analyses on the comparative sensing of SARS-CoV-2 mRNA by the intracellular TLRs of humans″. *Journal of Medical Virology*, 93(4), 2476-2486, 2021.

[7] Y. Hu, W. Li, T. Gao, and et al., ″ The severe acute respiratory syndrome coronavirus nucleocapsid inhibits type I interferon production by interfering with TRIM25-mediated RIG-I ubiquitination″. *Journal of virology*, 91(8), e02143-16, 2017.

[8] D. Yang, T. Geng, A. Harrison, and et al, ″Differential roles of RIG-I-like receptors in SARS-CoV-2 infection″. bioRxiv: the preprint server for biology, 02.10.430677. https://doi.org/10.1101/2021.02.10.430677, 2021.

[9] T. Yamada, S. Sato, Y. Sotoyama, and et al, ″RIG-I triggers a signaling-abortive anti-SARS-CoV-2 defense in human lung cells″. *Nature Immunology*, 1-9, 2021.

[10] X. Yin, L. Riva, Y. Pu, and et al, ″MDA5 governs the innate immune response to SARS-CoV-2 in lung epithelial cells″. *Cell reports*, 34(2), 108628, 2021.

[11] N. Krieger, J. Chen, and P. Waterman, ″Excess mortality in men and women in Massachusetts during the COVID-19 pandemic″. *The Lancet*, 395(10240), 1829, 2020.

[12] D. Gemmati, B. Bramanti, M. Serino, and et al, ″COVID-19 and individual genetic susceptibility/receptivity: role of ACE1/ACE2 genes, immunity, inflammation and coagulation. Might the double X-chromosome in females be protective against SARS-CoV-2 compared to the single X-chromosome in males? ″. *International journal of molecular sciences*, 21(10), 3474, 2020.

[13] Q. Bi, Y. Wu, S. Mei, and et al, ″Epidemiology and transmission of COVID-19 in 391 cases and 1286 of their close contacts in Shenzhen, China: a retrospective cohort study″. *The Lancet Infectious Diseases*, 20(8), 911-919, 2020.

[14] S. Jakhmola, B. Baral, and H. Jha, ″A comparative analysis of COVID-19 outbreak on age groups and both the sexes of population from India and other countries″. *The Journal of Infection in Developing Countries*, 15(03), 333-341, 2021.

[15] P. Bauer, J. Brugger, F. Koenig, and et al, ″An international comparison of age and sex dependency of COVID-19 Deaths in 2020-a descriptive analysis″. MedRxiv, 2021.

[16] A. Whiteman, A. Wang, K. McCain, and et al, ″Demographic and Social Factors Associated with COVID-19 Vaccination Initiation Among Adults Aged ≥65 Years - United States, December 14, 2020-April 10, 2021. MMWR″.*Morbidity and mortality weekly report*, 70(19), 725–730.https://doi.org/10.15585/mmwr.mm7019e4, 2021.

[17] F. Zheng, C. Liao, Q. Fan, and et al, ″Clinical characteristics of children with coronavirus disease 2019 in Hubei, China″. *Current medical science*, 40(2), 275-280, 2020.

[18] D. Bortolotti, V. Gentili, S. Rizzo, and et al, ″TLR3 and TLR7 RNA Sensor Activation during SARS-COV-2 Infection″. *Microorganisms*, 9(9), 1820, 2021.

[19] I. Lee, C. Wang, M. Lin, and et al, ″Effective strategies to prevent coronavirus disease-2019 (COVID-19) outbreak in hospital″. *The Journal of hospital infection*, 105(1), 102, 2020.

[20] S. Mukherjee, S. Huda, and S.SinhaBabu, ″Toll-like receptor polymorphism in host immune response to infectious diseases: A review″. *Scandinavian journal of immunology*, 90(1), e12771, 2019.

[21] A. Totura, A. Whitmore, S. Agnihothram, and et al, ″Toll-like receptor 3 signaling via TRIF contributes to a protective innate immune response to severe acute respiratory syndrome coronavirus infection″. *MBio*, 6(3), e00638-15, 2015.

[22] I. Biswas and G. Khan, ″Coagulation disorders in COVID-19: role of toll-like receptors″. *Journal of Inflammation Research*, 13, 823, 2020.

[23] S. Jeong, Y. Heo, J. Jeong, and et al, ″COVID-19 Subunit Vaccine with a Combination of TLR1/2 and TLR3 Agonists Induces Robust and Protective Immunity″. *Vaccines*, 9(9), 957, 2021.

[24] D. Kolli, T. Velayutham, and A. Casola, ″Host-viral interactions: role of pattern recognition receptors (PRRs) in human pneumovirus infections″. *Pathogens*, 2(2), 232-263, 2013.

[25] L. Xu, W. Wang, Y. Li, and et al, ″RIG-I is a key antiviral interferon-stimulated gene against hepatitis E virus regardless of interferon production″. *Hepatology*, *65*(6), 1823-1839, 2017.

[26] S. Sato, K. Li, T. Kameyama, and et al, ″The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus″. *Immunity*, 42(1), 123-132, 2015.

[27] L. Thorne, A. Reuschl, L. Zuliani-Alvarez, and et al, ″SARS-CoV-2 sensing by RIG-I and MDA5 links epithelial infection to macrophage inflammation″. *The EMBO Journal*, 40(15), e107826, 2021.

[28] J. García-Valero, J. Olloquequi, J. Montes, and et al, ″Deficient pulmonary IFN-β expression in COPD patients″. *PloS one*, 14(6), e0217803, 2019.

[29] I. Sola, F. Almazan, S. Zuniga, and et al, ″ Continuous and discontinuous RNA synthesis in coronaviruses″. *Annual review of virology*, 2, 265-288, 2015.

[30] R. Rangan, I.Zheludev, R. Hagey, and et al, ″RNA genome conservation and secondary structure in SARS-CoV-2 and SARS-related viruses: a first look″. *Rna*, 26(8), 937-959, 2020.

[31] G. Zhu, Y. Xu, X. Cen, and et al, ″Targeting pattern-recognition receptors to discover new small molecule immune modulators″. *European Journal of Medicinal Chemistry*, 144, 82-92, 2018.

[32] Q. Ruan, K. Yang, W. Wang, and et al, ″Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China″. *Intensive care medicine*, 46(5), 846-848, 2020.

[33] R. Channappanavar, and S. Perlman, ″Pathogenic human coronavirus infections: causes and consequences of cytokine storm and immunopathology″. *In Seminars in immunopathology* (Vol. 39, No. 5, pp. 529-539). Springer Berlin Heidelberg, 2017.

[34] P. Mehta, D. McAuley, M. Brown, and et al, ″ COVID-19: consider cytokine storm syndromes and immunosuppression″. *The lancet*, 395(10229), 1033-1034, 2020.

[35] K. Chen, F. Xiao, D. Hu, and et al, ″SARS-CoV-2 nucleocapsid protein interacts with RIG-I and represses RIG-mediated IFN-β production″. *Viruses*, 13(1), 47, 2021.

[36] K. Siu, K. Kok, M. Ng, and et al, ″Severe acute respiratory syndrome coronavirus M protein inhibits type I interferon production by impeding the formation of TRAF3· TANK· TBK1/IKKϵ complex″. *Journal of Biological Chemistry*, 284(24), 16202-16209, 2009.

[37] Y. Liu, D. Olagnier and R. Lin, ″Host and viral modulation of RIG-I-mediated antiviral immunity″. *Frontiers in immunology*, 7, 662, 2017.

[38] E. Mortaz, P. Tabarsi, M. Varahram, and et al, ″The immune response and immunopathology of COVID-19″. *Frontiers in immunology*, 11, 2020.