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Gene expression and polymorphisms of toll-like receptor 8 among Iraqi COVID-19 patients

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Abstract

Toll-like receptors trigger both innate and adaptive immune responses against viral infections by inducing the release of inflammatory cytokines and antiviral mediators. This study aims to examine the role of *TLR8* and genetic variants thereof in the progression and recovery of COVID-19 disease. It was conducted on 90 patients, (including 45 severe-critical cases, 45 mild-moderate cases),50 recovered cases, and 89 healthy people using quantitative real-time and allele-specific PCR for gene expression and gene polymorphisms determination, respectively. TLR8 gene expression did not show a significant difference among the study groups, while its median level showed statistically significant differences with respect to disease severity and Ct values. Interestingly, it showed a negative correlation with respect to the concentrations of some clinical parameters, which constituted a significant difference with respect to C-reactive protein and lactate dehydrogenase. However, in COVID-19 patients, low frequencies of the *TLR8* G and C alleles of rs3764879 in male patients and the G allele of rs3764880 in female patients were seen. Additionally, the A allele of rs37648801 in male patients and the A allele and its AA genotype in female patients increasingly affected their frequency. An association between males and females regarding increased frequency of the A allele of rs3764880 in patients and the G allele of rs3764880 in healthy subjects, constituting a risk factor and a protective factor, respectively, in both sexes. The frequency of certain alleles was found to be associated with either increased or decreased susceptibility to COVID-19 infection and severity. However, the TLR family has been shown to exhibit a particular kind of extremely accurate gene regulation.

Keywords: COVID-19, gene expression and gene polymorphisms of TLR8, inflammatory markers, Cycle threshold (Ct).

التعبير الجيني وتعدد اشكال مستقبلات شبيه التول 8 لدى مرضى كوفيد-19 من العراقيين

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الخلاصة

تعمل مستقبلات TLR8 الشبيهة بالتول على تحفيز الاستجابات المناعية الفطرية والتكيفية ضد العدوى الفيروسية عن طريق تحفيز إطلاق السيتوكينات الالتهابية والوسطاء المضادين للفيروسات. تهدف هذه الدراسة إلى دراسة دور TLR8 والمتغيرات الجينية منه في تطور مرض كوفيد-19 والتعافي منه.. أجريت الدراسة الحالية على 90 مريضاً (منهم 45 حالة شديدة- حرجة، و45 حالة خفيفة- متوسطة)، 50 حالة شفاء و(89) من الأصحاء. لم يظهرالتعبير الجيني لل TLR8 فرقا معنويا بين مجاميع الدراسة، بينما اظهر فروق ذات دلالة إحصائية فيما يتعلق بخطورة المرض وقيم Ct. ومن المثير للاهتمام، اظهاره علاقة سلبية فيما يتعلق بتركيزات بعض المعلمات السريرية، والتي شكلت فرقًا معنويا فيما يتعلق بالبروتين التفاعلي C ونازعة هيدروجين اللاكتات. من ناحية أخرى، تم تسجيل انخفاض تردد أليلات G و C لـ rs3764879 في المرضى الذكور وأليل G لـ rs3764880 في المرضى الإناث. بالإضافة إلى ذلك، فإن الأليل rs3764880 A في المرضى الذكور والأليل A والنمط الوراثي AA في المرضى الإناث أثر على تواترهم بطريقة متزايدة. كما وجد هناك ترابط بين الذكور والإناث فيما يتعلق بزيادة تواتر أليل Aلل rs3764880 في المرضى وأليل G لل rs3764880 في الأشخاص الأصحاء، مما يشكل عامل خطر وعامل وقائي على التوالي في كلا الجنسين. لذلك، وُجِد أن تردد بعض الأليلات مرتبط إما بزيادة أو انخفاض قابلية الإصابة بعدوى كوفيد-19 وشدتها. ومع ذلك، فقد ثبت أن عائلة TLR تظهر نوعًا معينًا من التنظيم الجيني العالي الدقة.

Introduction

On March 11, 2020, following the emergence of SARS-CoV-2, the World Health Organization declared the novel coronavirus disease, COVID-19, a global pandemic. The virus has a significant potential for spreading to other persons and can develop in as little as two days [1].

It was reported that an Iranian student who had been to Iraq on February 24, 2020, contracted COVID-19 for the first time. On February 25, an additional four cases from the same family in Kirkuk Governorate were reported; all of these individuals had also traveled recently to Iran. On February 27, a new case was announced in Baghdad related to a patient who had recently traveled to Iran [2].

It is well-established that the specific genes present in an organism's genome play a crucial role in determining disease-causing characteristics and susceptibility to various illnesses [3]. Therefore, through the production of pro-inflammatory cytokines and antiviral mediators, toll-like receptors (TLRs) enhance the innate and adaptive immune system's defense against viruses. On the other hand, pathogen-associated molecular patterns (PAMPs) produced by pathogenic microorganisms are recognized, and they manage the cytokine production necessary for the development of mature functional immunity [4].

It is well recognized that TLR allelic polymorphisms cause a variety of immunopathological effects when viruses infect a host [5]. Given the limited number of local studies on COVID-19, comprehensive research into all aspects of this topic is of paramount importance, none of them addressed what our current study addressed, as one of these studies investigated how TLR3 and RIG-I genes contribute to increased susceptibility to COVID-19 [6]. While another study focused on knowing the importance of the *IFIH* gene (rs11897697 and rs10203640) and how it contributes to susceptibility to infection with the Coronavirus [7].

Consequently, as far as we are aware, no prior work, examinations, or regional analyses have delved into the critical issue addressed within this current research endeavor.

Consequently, the goal of our current research is to shed light on the relationship between *TLR8* gene expression and polymorphisms and inflammatory markers in COVID-19 patients. By doing so, we hope to gain a deeper understanding of the pathophysiology of this illness and determine whether genetic polymorphisms and increased *TLR8* gene expression could either benefit patients by reducing disease severity or have the opposite effect by escalating the disease by triggering a cytokine storm.

Materials and methods

Populations studied

From November 15, 2022 to March 15, 2023, a total of 229 blood samples were collected for the current study. The samples were obtained from the Al-Shifa Center in Al-Zahraa Teaching Hospital located in Kut city, as well as from several other cities within the Wasit Governorate. These samples were taken from patients whose disease severity varied, from people who recovered from the disease three weeks after a positive real-time polymerase chain reaction (qRT-PCR) diagnosis at Al Karama Teaching Hospital in Kut, and from people who have never experienced illness or had the vaccine for this disease.

The experimental part of the study was carried out at the Kut National Central Public Health Laboratory. Subjects' serum ferritin, D-dimer, lactate dehydrogenase (LDH), weight, and blood sugar levels were measured, as well as C-reactive protein (CRP) levels. IgG and IgM levels were measured and blood groups were determined for each research group.
To investigate TLR8 gene expression using qRT-PCR, RNA was purified from the above groups. Furthermore, DNA was extracted to investigate gene polymorphism by allele-specific PCR of these research groups in the labs of the Iraqi Center for Cancer and Medical Genetics Research and the biology department labs at Baghdad University's College of Science. The WHO's guidelines for COVID-19 infections divided cases into three categories: recovered, severe-critical, and mild-moderate [8]. There were 50 recovered, 89 control, 45 severe-critical, and 45 mild-moderate cases in this study. Age groups, sex, and body mass index (BMI) were also taken into consideration in this study.

It was necessary for all of the COVID-19 participants who recovered to have gotten the virus at some time in the past; also, their IgM and qRT-PCR test results had to be negative while their IgG test results had to be positive for the COVID-19 virus. In the seemingly healthy patients, the COVID-19 antibodies (IgM and IgG) and qRT-PCR findings were negative; however, in the groups classified as mild-moderate and severe-critical, the COVID-19 antibodies (IgM) and qRT-PCR results were positive. Samples younger than ten years old and samples from the same family were excluded. Participants in the study ranged in age from 20 to 90, with (99) males and (130) females. The ill group, the recovered group, and the healthy group had mean ages ± standard deviations of 47.9 ± 17.8, 38.1 ± 14.7, and 37.2 ± 14.1 years, respectively.

Laboratory tests

Five-milliliter blood samples and nasopharyngeal or oropharyngeal swabs were collected from the study groups. The swabs were placed straight into viral transport medium (VTM). To isolate the viral RNA, a QIA amp Viral RNA Mini kit (Qiagen Germany) was utilized. SARS-CoV-2 was identified via real-time polymerase chain reaction analysis, and the manufacturer's instructions were adhered to. To separate the blood samples, three tubes containing gel, sodium citrate, and EDTA were utilized. Plasma D-Dimer analysis was performed on the blood samples (sodium citrate tubes), which were centrifuged for 20 minutes at 3500 rpm, using a specialized automated protein analyzer (BIOSENCER STANDARD F200 analyzer) supplied by Suwonsi, Gyeonggi Co., Ltd. Korea

2020.

To obtain serum for measuring ferritin, lactate dehydrogenase (LDH), and C-reactive protein levels, the second part of the blood samples collected in gel tubes underwent centrifugation for 10 minutes at 6000 rpm to separate the serum. Using the Roche Cobas Integra 400 plus electro-chemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany), the serum concentrations of LDH and CRP were assessed. The Ferritin was also evaluated using a miniVIDAS analyzer (ELFA) from BioMerieux, following the manufacturer's instructions. After being collected in EDTA tubes, 200 microliters of the blood samples were quickly transferred to a tube holding 600 µl of triazole, thoroughly mixed, and stored at -20°C. After that, TLR8 gene expression was determined by extracting RNA from this tube. To explore TLR8 gene polymorphisms, the remaining volume of EDTA blood was stored at −20°C for DNA extraction.

Real-Time- Quantitative PCR for Gene Expression

The TransZOl Up plus RNA kit (Transgen, China) was used to extract RNA from the blood tube containing Triazole (according to the manufacturer's instructions) of the research groups' cases. Complementary DNA (cDNA) synthesis was performed using the EasyScript® two-Step gDNA deletion and complementary DNA creation Super-Mix (Transgen, China). Following the addition of RNA (3-5 µl), 1 µl of Random Primer (0.1µg/µl) and 1 µl of Anchored Oligo (dT) Primer (0.5µg/µl) to RT-PCR tubes, the tubes were incubated in a thermocycler for ten minutes at 4 °C and five minutes at 65 °C. It was then added to a final amount of 20µl after incubation, which contained 3µl RNase-free water, 1µl Easy Script®RT/RI enzyme mix, 1µl gDNA remover, and 10µl EX reaction mix. A thermocycler was used for 10 minutes at 25 °C, 30 minutes at 42 °C, and 5 seconds at 85 °C. To amplify cDNA, the TransStart® Green qPCRSuper mix kit (Transgene, China) and certain primers were utilized. The TLR8 gene (forward-5-TGTGATGGTGGTGCTTCAAT-3 and reverse-5-ATGCCCCAGAGGCTATTTCT-3), [9] and Beta-actin (Housekeeping genes) (forward-5-GAAGGATTCCTATGTGGGCG-3 and reverse-5 TGGTGGTAAAGCTGTAC-3) [10]. The reaction included 10 µl of the master mix SybrGreen, 1 µl of each primer, 3 µl of cDNA, and 5 µl of nuclease-free water. Amplification was first denaturized at 94ºC for 30 minutes, then 40 cycles of 94ºC (5 sec), 52ºC (15 sec) for the TLR8 gene, and 56ºC (15 sec) for the housekeeping gene, and 72ºC (20 sec). The final dissociation parameters used in the study were a temperature range of 55°C to 95°C and a cycle duration of 1 minute.

Calculation of Folding rate

The level of *TLR7* gene folding rate was calculated as follows:1. ΔCT = CT target gene - CT housekeeping gene (for each patient and control specimen), 2- ΔΔCT (each specimen) = ΔCT patient specimen-ΔCT average control specimens. 3- Fold of gene expression (each specimen) = 2^- ΔΔCT, Folding rate = 2^- ΔΔCT average of patients/ 2^- ΔΔCT average of controls. CT stands for threshold cycle [11].

Conventional PCR for TLR8 gene polymorphism

Three TLR8 SNPs (rs3764789, rs3764880, and rs5744080) were identified. Using the gSYNCTMDNA extraction kit (Geneaid, Taiwan), genomic DNA was extracted from EDTA blood. This work designed the primers that were utilized in the conventional PCR amplification process. Additionally, target SNPs were found using polymerase chain reaction (PCR) analysis on isolated DNA following assessments of purity and concentration. A total of 25 µL was used for the PCR, and 5 µL of AccuPower PCR PreMix (Bioneer, Korea) was used, 1 µL forward primer (5'-GTAAACTTCTGTAAAACACACGCTA[C/G]-3′), 1 µL reverse primer (5'-TCATAGCAAGCCGCTTTACC -3′) (Annealing Tm 53.5 ◦C) of 3764879;1 µL forward

primer (5'-GGAATGAAAAATTAGAACAACAGAAAC[A/G]-3′), 1 µL reverse primer (5'-TTCCCTCACCTTCTCTTCCA -3′) (Annealing Tm 53◦C) of rs3764880;1 µL forward primer (5'-ATCACTATCTTTCAATTCTCTTTCACA[C/G]-3′), 1 µL reverse primer (5'-CAATGCCCGTAGAGACAAAA -3′) (Annealing Tm 52.5 ◦C) of rs5744080, and 15 µL deionized distilled water, tracking dye, and 3 µL DNA template. The tube was moved to a thermal cycler (Eppendorf, Germany) that was set up to operate under the following optimal settings: 94 °C for 5 minutes for initial denaturation, 35 cycles for denaturation (94 °C for 30 s), annealing (as described above for 30 s), and extension (72 °C for 45 s), and a final cycle for extension (72 °C for 5 min), 1.5% agarose gel was used for the electrophoresis of the PCR results. After staining with ethidium bromide dye solution (4%), target bands of certain primers were visible together with DNA ladder bands (100 bp) for 55 minutes at 5 v/cm2 under a UV light transilluminator at 320–380 nm.

Statistical analysis

The normality of continuous variables was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Standard deviation (SD) and mean were used to describe normally distributed variables, and the Student t-test was used to assess significant differences. The median and interquartile range (IQR) were employed to express nonparametric variables, and the Mann-Whitney U test was utilized to identify significant differences. The Pearson correlation (r) and Spearman rank-order correlation coefficient (rs) were used to explain the type and strength of the relationship between the variables. The statistical software Graph Prism version 8.0 (San Diego, California USA) and SPSS version 25.0 (Armonk, NY: IBM Corp.) were used for this analysis. The SNPs under investigation were provided as genotyping frequencies and alleles. Using a method called direct gene counting, allele frequency was determined. SNP genotype frequencies were evaluated by Hardy-Weinberg equilibrium (HWE) using an online calculator (https://www.had2know.org/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html) through Pearson's Chi-square test. The association between SNPs and disease was assessed using the odds ratio (OR) and its corresponding 95% confidence interval (CI). These estimates were obtained with WinPepi program version 11.65. A p-value of less than 0.05 was deemed significant.

Result

Gene expression of TLR8 in qRT-PCR

The current study's results revealed that the ratio of relative *TLR8* gene expression was 1.21 in the group of COVID-19 patients compared to healthy control individuals (Fold median (IQR) is 7.6 (1.03 – 14.04) in the patient group and 6.3 (4.38 – 13.71) in the healthy control individuals), while the ratio was 1.25 in the group of patients compared to the recovered group (Fold median is 6.1 (3.54 – 10.9 in the recovered group). This indicates that there are no statistically significant differences between these three groups in terms of the receptor's gene expression ($p = 0.861$) [Figure-1].

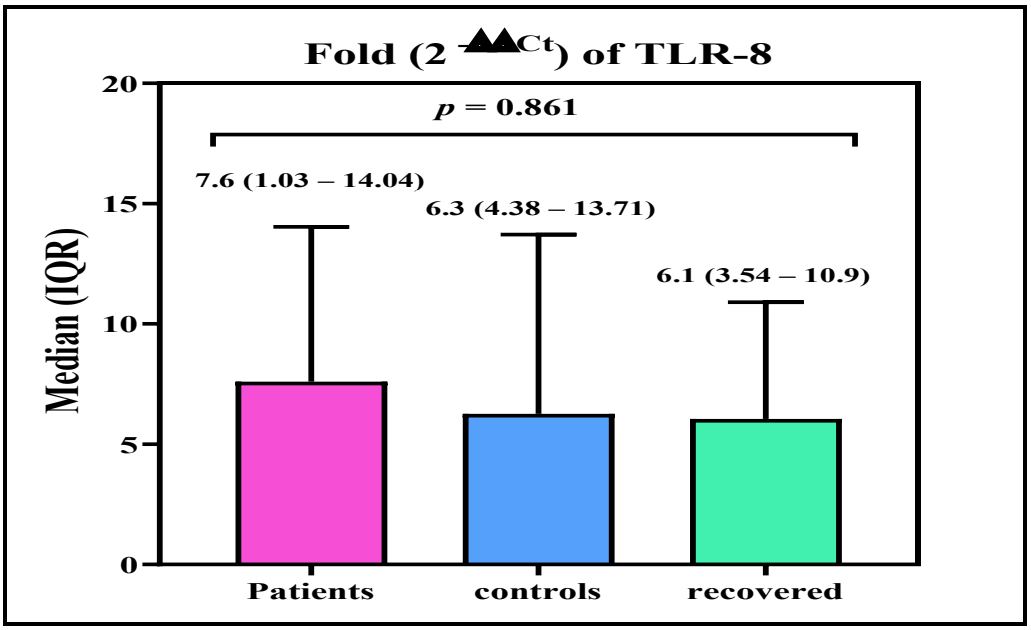

Figure 1: Fold rate of TLR8 in the COVID-19 patient group, the recovered group, and the healthy control group.

Relatedly, a statistically significant difference was found in the severity of the disease among patient groups, [8.6 (6.4 – 14.4) in the mild-moderate patient group *vs.* 2.5 (0.34 – 11.9) in the sever-critical patient group, $p = 0.011$] when the median levels of TLR8 fold were stratified according to the characteristics of the COVID-19 patient group, the recovered group, and the healthy control group. In relation to the Ct values, which are classified as high viral loads (16–26) and low viral loads (27–36), the median TLR8 fold levels were [2.9 (0.38 – 11.8) *vs.* 8.5 (6.4 – 15.5) respectively, $p = 0.024$]. In contrast, there were no statistically significant differences observed in any of the three groups with respect to blood type, age, sex, body mass index, or diabetes [Table-1].

Table 1: Median levels of TLR8 fold stratified according to characteristics of the COVID-19 patient group, the recovered group, and the healthy control group.

Character		**Fold ($2^{-\Delta\Delta Ct}$) *TLR8* median (IQR)**		
		Patient (no. 90)	**Recovered (no. 50)**	**Control (no. 89)**
Age group	≤ 45	8.6 (3.5 – 14.2)	5.4 (3.1 – 8.7)	6.9 (3.5 – 9.8)
	> 45	3.9 (0.4 – 8.6)	7.5 (5.8 – 28.8)	5.5 (4.5 – 10.9)
	p-value	$p = 0.071$	$p = 0.067$	$p = 0.722$
Sex	Male	7 (0.98 – 11.2)	6.6 (5.4 – 19.3)	5 (3.5 – 7.02)
	Female	8.3 (2.6 – 14.9)	5.7 (2.5 – 9)	6.9 (3.6 – 11.6)
	p-value	$p = 0.563$	$p = 0.346$	$p = 0.188$
Severity	Mild-moderate	8.6 (6.4 – 14.4)	NA	NA
	Severe	2.5 (0.34 – 11.9)		
	p-value	**$p = 0.011$**		
Ct value	16 – 26	2.9 (0.38 – 11.8)	NA	NA
	27 - 36	8.5 (6.4 – 15.5)		
	p-value	**$p = 0.024$**		
Diabetes mellitus	Yes	*4.8 (0.38 – 8.3)*	6.2 (5.8 – 6.3)	NA
	No	8.2 (1.97 – 14.4)	6.9 (4.1 – 18.6)	
	p-value	$p = 0.147$	$p = 0.917$	
BMI	NW	7.9 (1.1 – 17.7)	5.6 (4.1 – 8.4)	5.5 (2.5 – 8.6)

	OW	*6.6 (0.96 – 11.8)*	6.9 (4.8 – 19.3)	9.8 (4.8 – 11.3)
	p-value	$p = 0.348$	$p = 0.240$	$p = 0.113$
Blood group	A	5.7 (0.96 – 12.8)	6.9 (5.1 – 8.9)	5.9 (3.3 – 11.4)
	B	5.8 (1.1 – 13.6)	5.1 (1.5 – 6.2)	5.5 (3.5 – 13.2)
	O	8.3 (3.2 – 17.7)	7.6 (5.6 – 18.6)	8.8 (6.1 – 11.6)
	AB	8.3 (0.38 – 11.8)	6.9 (2.5 – 20.2)	6.5 (2.4 – 9.8)
	p-value	$p = 0.870$	$p = 0.576$	$p = 0.583$

IQR: Interquartile range; NA: Not applicable; NW: normal weight, OW: overweight/obese; p: Kruskal-Wallis test and Mann-Whitney U test probability.

A Pearson correlation analysis of scatter plots revealed a negative correlation between *TLR8* gene expression in COVID-19 patients and the concentrations of CRP, ferritin, D-dimer, and LDH. This resulted in statistically significant differences between the gene expression of this receptor and both CRP (rs = - 0.325; $p = 0.011$) and LDH (rs = - 0.243; $p = 0.007$), albeit not with reference to the other two parameters [Figure-2].

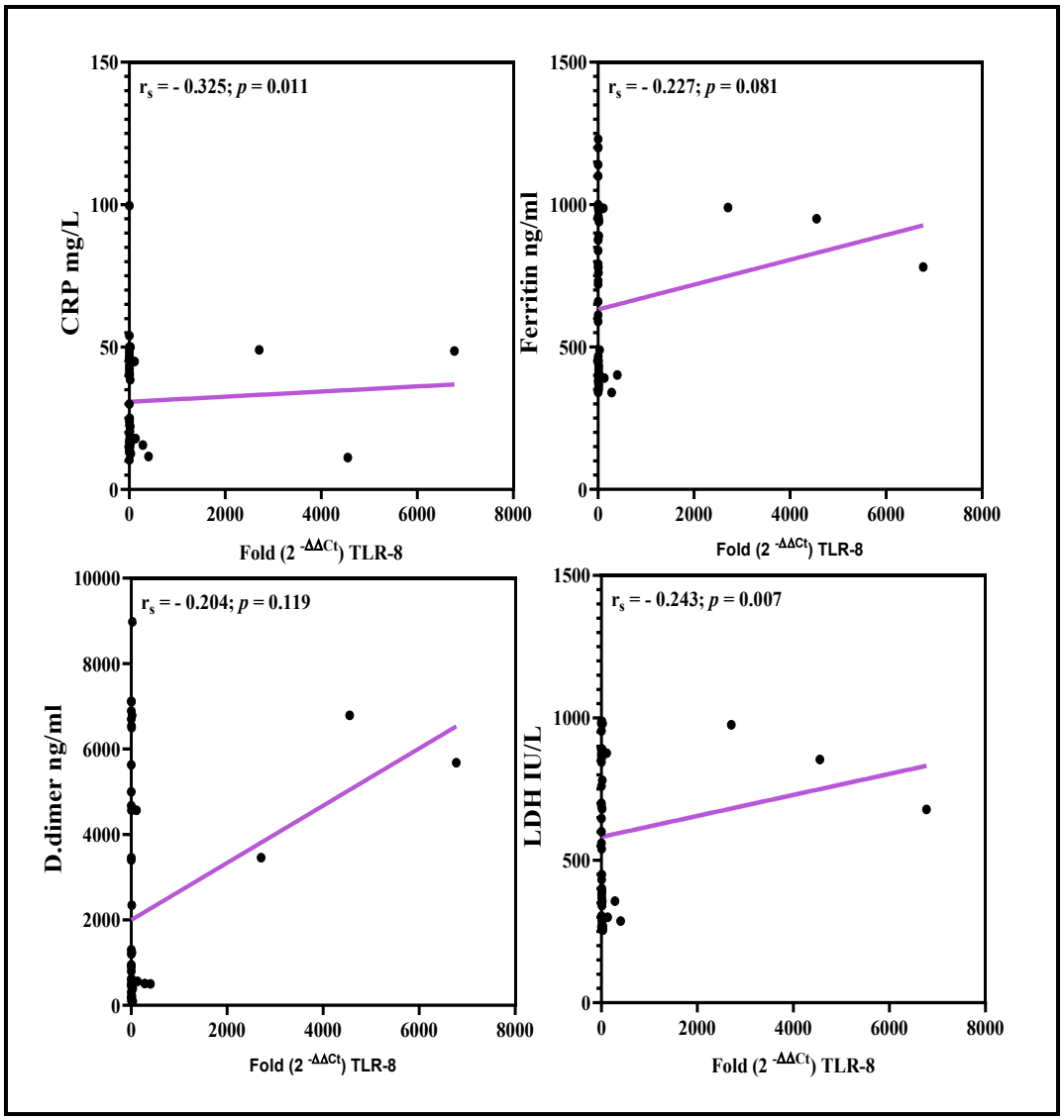

Figure 2: Scatter plot Spearman rank-order correlation coefficient (r_s) was used to analyze the relationship between TLR8 gene expression in COVID-19 patients and CRP, ferritin, D. dimer, and LDH.

Single nucleotide polymorphisms of TLR8 gene with study populations

The results of electrophoresis for PCR product showed bands of different molecular sizes were encountered [Figure-3].

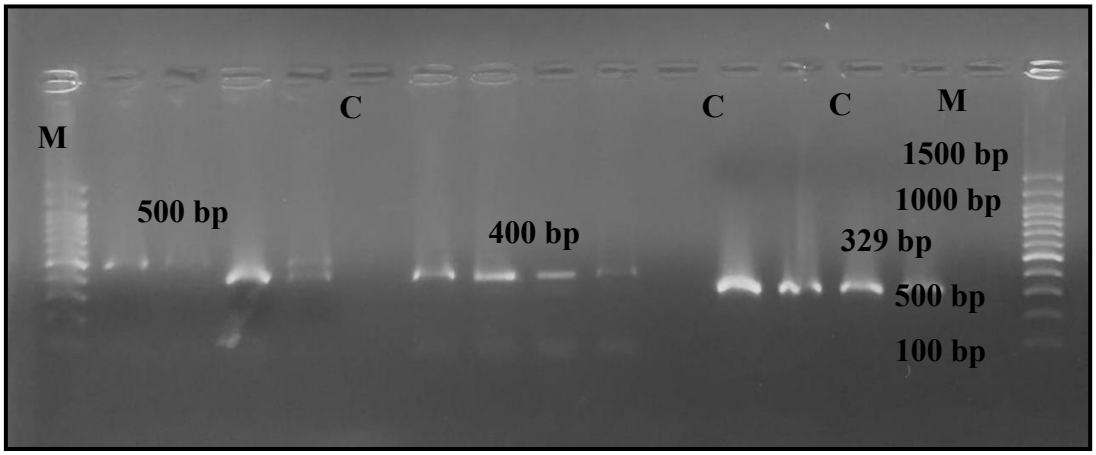

Figure3 :- Representative images of agarose gel electrophoresis (1.5%; 5 V/cm2 for 5 0 minutes) of DNA–PCR products for *TLR8* gene SNPs; **500** bp for rs5744080 (C/G), 400 bp for rs3764880 (G/A), 329 bp for rs3764879 (G/C). M: DNA ladder (100bp); C: Negative control.

Baseline characteristics of investigated samples

The present study showed no significant difference in *TLR8* gene SNP, rs3764879 G and C allele ($p = 0.121$), while it showed a significant difference in the *TLR8* gene SNPs rs3764880, 52.4% for male patients and 88.9% for the males control subjects, for the dominant G allele, while 47.6% for male patients and 11.1% for the males control subjects, OR 7.3 for the co-dominant (recessive) A allele ($p = 0.001$), and rs5744080 C allele, 61.9% for male patients and 94.4% for the males control subjects, for the dominant C allele, while 38.1% for male patients and 5.6% for the males control subjects, OR 10.5 for the co-dominant (recessive) G allele ($p = 0.001$) among male patients (n = 42) and male controls subjects (n = 36) of COVID-19 disease (Table-2).

Table 2: Single nucleotide polymorphisms of TLR8 gene in male COVID-19 patients and healthy controls.

TLR8 gene SNPs	Allele	male patients (N = 42)		males control (N = 36)		OR	95% CI	*p*-value
		N	%	N	%			
rs3764879	G	28	66.7	30	83.3	0.4	0.2-1.2	0.121
	C	14	33.3	6	16.7	2.5	0.9-7.3	
rs3764880	G	22	52.4	32	88.9	0.2	0.04-0.5	**0.001**
	A	20	47.6	4	11.1	7.3	2.2-23.8	
rs5744080	C	26	61.9	34	94.4	0.1	0.02-0.5	**0.001**
	G	16	38.1	2	5.6	10.5	2.3-48.6	

CI confidence interval, N: absolute number, OR: odds ratio, *p*: two-tailed Fisher's probability (significant *p*-value is indicated in bold), SNP: single nucleotide polymorphism, TLR: Toll-like receptor.

Based on the information provided, the current study found no significant difference in the TLR8 gene SNP when comparing the results between females and the other group examined, rs3764879 G/C allele and their genotypes and rs5744080 C/G allele and their genotypes except CG genotype which shows a slightly significant differences between female patients and

female control (*p*= 0.051), while it showed a significant difference in TLR8 gene SNPs rs3764880 G/A allele, 45.8 % for female patients and 66 % for the females control subjects, for G allele and 54.2 % for female patients and 34 % for the female control subjects, for A allele, OR 2.3 (*P*=0.005) , and their genotype AA, 37.5% for female patients and 15.1 % for the female control subjects, OR 4.0 (*P*= 0.012) among female patients (n = 48) and female controls (n = 53) of COVID-19 disease (Table-3).

Table 3: Single-nucleotide polymorphisms of *TLR8* gene in female COVID-19 patients and healthy controls.

TLR8 SNPs	Allele/ genotype	female patients (N = 48)		female control (N = 53)		OR	95% CI	*p*-value
		N	%	N	%			
rs3764879 G/C	G	80	83.3	78	73.6	Reference		
	C	16	16.7	28	26.4	0.6	0.3 – 1.1	0.124
	GG	34	70.8	29	54.7	Reference		
	GC	12	25.0	20	37.7	0.5	0.2 – 1.2	0.192
HWE-*p*-value	CC	2	4.2	4	7.6	0.4	0.1 – 2.2	0.416
				0.831				
rs3764880 G/A	G	44	45.8	70	66	Reference		
	A	52	54.2	36	34	2.3	1.3 – 4.0	**0.005**
	GG	14	29.2	25	47.2	Reference		
	GA	16	33.3	20	37.7	1.4	0.6 – 3.6	0.487
HWE-*p*-value	AA	18	37.5	8	15.1	4.0	1.4 – 11.4	**0.012**
				0.248				
rs5744080 C/G	C	55	57.3	67	63.2	Reference		
	G	41	42.7	39	36.8	1.3	0.7 – 2.3	0.472
	CC	12	25.0	22	41.5	Reference		
	CG	31	64.6	23	43.4	2.5	1.0 – 6.0	**0.051**
HWE-*p*-value	GG	5	10.4	8	15.1	1.2	0.3 – 4.1	1.000
				0.626				

SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; N: absolute number; OR: Odds ratio; CI: Confidence interval; p: Two-tailed Fisher's exact probability; p: probability (significant p-value is indicated in bold).

On the other hand, none of the *TLR8* gene SNPs, rs3764879 G and C allele, rs3764880 G and A allele and rs5744080 C and G allele showed any statistical significance between the group of recovered males and the group of healthy control males of COVID-19 (Table -4).

Table 4: Single nucleotide polymorphisms of TLR8 gene in males of recovered and control groups.

TLR8 gene SNPs	Allele	males recovered group (N = 21)		male control group (N = 36)		OR	95% CI	*p*-value
		N	%	N	%			
rs3764879	G	16	76.2	30	83.3	0.6	0.2-2.4	0.511
	C	5	23.8	6	16.7	1.6	0.4-5.8	
rs3764880	G	19	90.5	32	88.9	1.2	0.2-6.9	1.000
	A	2	9.5	4	11.1	0.8	0.2-4.9	
rs5744080	C	18	85.7	34	94.4	0.4	0.1-2.2	0.346
	G	3	14.3	2	5.6	2.8	0.5-17.9	

CI confidence interval, N: absolute number, OR: odds ratio, p: two-tailed Fisher's probability, SNP: single nucleotide polymorphism, TLR: Toll-like receptor

None of the examined TLR8 SNPs demonstrated statistically significant differences among the aforementioned alleles, except the TLR8 SNP rs3764879 G/C allele, 56.9 % for female recovered subjects and 73.6% for the females control subjects for G allele and 43.1% for female recovered subjects and 26.4 % for the females control subjects for C allele, OR 2.1, (*P*= 0.036) and for their genotype CC, 31.1 % for female recovered subjects and 7.6% for the females control subjects, OR 5.0 (*P*= 0.023) between females recovered (N=29) and healthy control subjects (N= 53) (Table -5).

Table 5: Single nucleotide polymorphisms of *TLR8* gene in females of recovered and healthy control groups.

TLR8 SNPs	Allele/ genotype	female Recovered subjects (N = 29)		Female Control subjects (N = 53)		OR	95% CI	*p*-value
		N	%	N	%			
rs3764879 G/C HWE-*p*-value	G C GG GC CC	33 25 13 7 9	56.9 43.1 44.8 24.1 31.1	78 28 29 20 4 0.831	73.6 26.4 54.7 37.7 7.6	Reference 2.1 Reference 0.8 5.0	 1.1 – 4.1 0.3 – 2.3 1.4 – 18.5	 **0.036** 0.788 **0.023**
rs3764880 G/A HWE-*p*-value	G A GG GA AA	37 21 12 13 4	63.8 36.2 41.4 44.8 13.8	70 36 25 20 8 0.248	66 34 47.2 37.7 15.1	Reference 1.1 Reference 1.4 1.1	 0.6 – 2.2 0.5 – 3.6 0.3 – 4.0	 0.864 0.621 1.000
rs5744080 C/G HWE-*p*-value	C G CC CG GG	44 14 16 12 1	75.9 24.1 55.2 41.4 3.4	67 39 22 23 8 0.626	63.2 36.8 41.5 43.4 15.1	Reference 0.6 Reference 0.7 0.2	 0.3 – 1.1 0.3 – 1.8 0.02 – 1.4	 0.117 0.631 0.127

SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; N: absolute number; OR: Odds ratio; CI: Confidence interval; p: Two-tailed Fisher's exact probability; p: probability (significant p-value is indicated in bold).

Concerning allele frequencies of *TLR8* genes SNPs stratified by clinical severity in males of COVID-19 patients, none of the SNPs for the above-mentioned alleles showed any significant differences, except the *TLR8* SNP rs5744080 C and G allele, 75% for mild-moderate cases patients and 42.3% for severe- critical cases patients, OR 4.8 for C allele and 25% for mild-moderate cases patients and 57.7% for severe- critical cases patients, OR 0.2 for G allele (P= 0.029) between the mild- moderate cases patients (N= 16) and sever-critical cases patients (N= 26) (Table-6).

Table 6: Allele frequencies of *TLR8* gene SNPs stratified by clinical severity in males of COVID-19 patients.

TLR8 gene SNPs	Allele	Clinical severity in males (N = 42)				OR	95% CI	*p*-value
		Mild-moderate (N =16)		Severe-critical (N =26)				
		N	%	N	%			
rs3764879	G	14	87.5	21	80.8	1.7	0.3-9.4	0.690
	C	2	12.5	5	19.2	0.6	0.1-3.4	
rs3764880	G	9	56.3	12	46.2	1.5	0.5-5.1	0.751
	A	7	43.7	14	53.8	0.7	0.2-2.3	
rs5744080	C	12	**75**	10	42.3	4.8	1.3-18.4	**0.029**
	G	4	25	16	**57.7**	0.2	0.1-0.8	

CI confidence interval, N: absolute number, OR: odds ratio, *p*: two-tailed Fisher's probability (significant *p*-value is indicated in bold), SNP: single nucleotide polymorphism, TLR: Toll-like Receptor.

With respect to the allele and genotype frequencies of *TLR8* gene SNPs categorized by clinical severity in female COVID-19 patients, none of the SNPs of the above-mentioned alleles and their genotypes showed any significant differences between the mild- moderate cases patients (N= 29) and the sever-critical cases patients (N= 19) (Table-7).

Table 7: Allele and genotype frequencies of *TLR8* gene SNPs stratified by clinical severity in female COVID-19 patients.

TLR8 SNPs	Allele/ genotype	Clinical severity in females (N = 48)				OR	95% CI	*p*-value
		Mild-moderate (N =29)		Severe-critical (N =19)				
		N	%	N	%			
rs3764879 G/C	G	51	87.9	30	78.9	Reference		
	C	7	12.1	8	21.1	0.5	0.2 – 1.5	0.262
	GG	22	75.9	12	63.2	Reference		
	GC	7	24.1	6	31.6	0.6	0.2 – 2.2	0.521
	CC	0	0	1	5.3	0.1	0.00 – 3.9	0.371
rs3764880 G/A	G	30	51.7	17	44.7	Reference		
	A	28	48.3	21	55.3	0.8	0.4 – 1.7	0.537
	GG	9	31	6	31.6	Reference		
	GA	12	41.4	5	26.3	1.6	0.4 – 6.6	0.712
	AA	8	27.6	8	42.1	0.7	0.2 – 2.6	0.722
rs5744080 C/G	C	38	65.5	18	47.4	Reference		
	G	20	34.5	20	52.6	0.5	0.2 – 1.1	0.093
	CC	11	37.9	2	10.5	Reference		
	CG	16	55.2	14	73.7	0.2	0.04 – 1.04	0.086
	GG	2	6.9	3	15.8	0.1	0.01 – 1.1	0.099

SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval; N: absolute number p: Two-tailed Fisher's exact probability; p: probability.

Discussion

As the first line of defense, the innate immune system recognizes the virus using pattern recognition receptors (PRRs), such as the TLR family, and initiates inflammatory pathways that facilitate the removal of the virus [12]. One of these families is *TLR8*, located on the human X chromosome [13]. *TLR8* gene expression is higher in the patient group than in the recovered and healthy groups, according to the current study, however this increase is not statistically significant [Figure 1]. At first glance this may appear to be poor gene expression for this receptor, but it is actually a form of fine-grained genetic regulation between different types of TLRs. In comparison to the groups of patients with mild-moderate illness and those with low

viral loads (C t value = 27–36), TLR8 increased significantly in each of these groups [Table 1]. The observed decrease in this receptor's gene expression may be attributed to an elevated viral load. In severe cases, this high viral burden can induce a substantial and dysregulated immune response, potentially leading to impaired immune system functioning. Despite the fact that certain research has demonstrated that *TLR8* gene expression is weaker than *TLR7* [14, 15]. From our vantage point, genetic regulation and compensation between distinct TLR types is the most likely explanation for this example and all other cases where *TLR8* gene expression did not show a substantial difference. A clear suppression of *TLR8*-induced cytokine production was seen upon the administration of the same ligand for both *TLR7* and *TLR8*, indicating that *TLR7* may have a modulatory influence on the *TLR8* response [16].

It is extremely unexpected that research on mice suggests that *TLR7* might adversely regulate *TLR8*, since *TLR8* expression was upregulated when *TLR7* was absent, pointing to a significant immune response compensatory mechanism [17]. Within the same context, a negative correlation was observed between *TLR8* gene expression and the concentrations of several classical parameters, including CRP, ferritin, D-dimer, and LDH. This negative correlation was highest and demonstrated a significant difference with respect to CRP and LDH [Figure 2]. This clearly confirms our earlier view of the previously discussed negative regulation of *TLR7* over *TLR8*. However, there was a discrepancy between the immunological response, as shown by *TLR8* gene expression, and the rise in concentrations of these crucial classical markers. The strong inverse relationship between *TLR8* gene expression and CRP and LDH supports this hypothesis even more. This indicates that the *TLR7* gene is really negatively regulating the *TLR8* gene in order to compensate.

The factors determining why certain individuals infected with COVID-19 remain asymptomatic while others experience severe illness are still not fully understood by researchers. The answer to this question and the identification of the variables influencing SARS-CoV-2 pathogenicity will be helpful in developing effective treatment plans and controlling infections [18, 19]. Despite the fact that the genetic background of the host has an impact on the severity of the disease and the prognosis of the patient [20, 21].

In addition to recognizing viral particles and initiating the innate immune system by secreting pro-inflammatory cytokines, toll-like receptors (TLRs) can cause persistent inflammation and tissue destruction in the host by activating the inflammasome and producing IL-1β, which in turn induces IL-6 and hyperactivates the immune system, which can lead to acute lung injury [22, 23]. TLRs have revolutionized the field of immunology since their discovery because they provide a link between the identification of pathogens by innate immune cells and the activation of the adaptive immune response. TLRs also assist in the activation of the adaptive immune system by overexpressing major histocompatibility complex on dendritic cells [24, 25]. Consequently, in viral infections, genetic variants and allelic polymorphisms of these receptors cause a number of immunopathological effects [26, 27].

Therefore, by identifying the association of TLR8 gene polymorphism with the emergence and susceptibility of COVID-19, the present study investigated three gene SNPs for *TLR8*, rs3764879, rs3764880, and rs5744080, that may play a part in defending against or raising vulnerability to coronavirus infection in Iraqi patients. This was done in order to better understand the role of *TLR8* in the etiology and pathogenesis of COVID-19.
For detections of these SNPs, allele-specific primer technique was used. It should be noted that these SNPs are located on the X chromosome:12,867,072-12,890,361, rs 3764879 G/C is located in promoter region, rs 3764880 G/A is located in start lost in Exon1, and rs 5744080 C/G is located in intron (https://www.ensembl.org/Homo_sapiens/Info/Index) . Because their

gene is on the X chromosome, all SNPs pertaining to this receptor in male individuals do not follow HWE equilibrium.

In male sex, it was noted that allele A on rs3764880 (*TLR8*) was prevalent in patients whereas on rs3764880 (*TLR8*) allele G was prevalent in healthy control individuals. On the rs5744080 (*TLR8*) polymorphic site, mutant allele G was prevalent among COVID-19 individuals as compared to healthy controls, while on rs5744080 (*TLR8*) allele C was prevalent in healthy control individuals. Which suggests that, depending on their increased frequency in healthy individuals or patients, these alleles either play a protective or the primary role in raising a person's susceptibility to contracting COVID-19 respectively (Table-2). The reason for the rise in the number of patients with risk alleles is that the SNPs altered the amino acid sequence in one way or another in the TLRs, or by altering splicing, or alternative splicing, if they are located in the intron site. These changes have an impact on transcription, translation, and other processes, which in turn makes these receptors less effective at triggering the proper immune response to eliminate pathogens. In contrast, the SNP that had a protective role undoubtedly led to an increase in the efficiency of the receptor in question, and thus a stronger, more organized and more precise immune response. Therefore, it is essential for accurately recognizing the role that TLRs play in the pathogenic processes associated with SARS-CoV-2 [28, 29].

The current research has demonstrated an association between increased susceptibility to COVID-19 and the allelic (A) and genotypic (AA) forms of *TLR8* rs3764880, suggesting that this SNP is a predisposing risk factor at these two levels in female patients. While the TLR8 SNP rs3764880 of G allele showed exactly the opposite, as it showed an increase in the number in healthy people compared to sick patients, indicating the protective role of this allele (Table -3). This highlighted the importance of A allele of the rs3764880 in SARS-CoV-2 disease, which indicates that this polymorphism certainly affects the work of this receptor towards increasing human susceptibility to the aforementioned disease [30].

This study showed that males are similar to females with regard to increase frequency of A allele of rs3764880 in patients and G allele of rs3764880 in healthy subjects, which constitutes a risk and a protective factor respectively in both sexes. This supports previous study on the allele's resemblance in both sexes, although other investigations demonstrate that this isn't the case. Given that human populations' genetic makeup can affect an infectious disease's propensity and outcome, particularly COVID-19[31]. Remarkably, more investigation identified two unique SNPs in the *TLR8* exon, rs5744080 and rs2159377; however, none was shown to influence the severity of symptoms in the COVID-19 patients [32, 33].

Despite the lack of direct supporting or contradictory data from the current investigation, which show that there are no statistically significant differences between the recovered group and the healthy control group for any of the SNPs of the indicated alleles in males (Table -4), these findings may be realistic, as some polymorphisms may have a role in increasing susceptibility to a particular disease, or vice versa by showing a protective role or increasing resistance to a particular treatment, and the like. Since the condition of recovered people does not differ greatly from healthy people, it does not seem logical to have a role for SNPs on allelic level. Therefore, this conclusion was consistent with the results of this study. In contrast to what was shown in males, notably, this investigation revealed that rs3764879 of *TLR8* was increased in recovered female individuals at C allele and CC genotype levels which may indicate its role in increasing the recovery rate of patients and getting rid of the virus, while rs3764879 showed exactly the opposite at the G allelic level, as it showed an increase in healthy females compared to recovered females, indicating a protective role (Table-5).

TLR8 rs3764880-G allele was associated with spontaneous HCV clearance in both sexes albeit with an apparently stronger association in males [34].

The rs5744080 C allele (*TLR8*) showed a significant increase in mild to moderately ill male patients compared to severely-critically ill male patients (Table-6). This suggests, in one way or another, the protective role of this allele through its role in increasing, improving, or regulating the immune response and making it more capable of confronting this disease by lessening the intensity of the illness in those who are affected, by boosting the stimulation of cell production and other immune components in a manner consistent with increasing the efficiency of the immune response in both types. According to a previous study on Dengue hemorrhagic fever (DHF), women patients were more likely to be TLR8-rs3764880-A/A if they had high monocyte and high IgG levels. Patients with DHF who had high IgG and low monocyte levels were more likely to have the TLR8-rs1548731-C/C and TLR8-rs5741883-C/C genotypes [35,36].

On the other hand, none of the individual alleles for both of the mentioned SNPs showed significant differences regarding the severity of the clinical disease in female COVID-19 patients (Table - 7), suggesting that there is no protective role or increased susceptibility to this disease that these alleles are able to play. Even so, there isn't any proof at this time to confirm or refute these results, but in any case, the alleles that were studied do not appear to have a role in the severity of this disease.

The promising results concerning TLR8 SNPs may have substantial implications for the field of genetically-based personalized treatment as they could lead to a better knowledge of their potential involvement in the development, outcome, and prevention of COVID-19 disease. Patients with COVID-19 may benefit from improved clinical care and clinical management if early death prediction is possible.

Conclusions

Due to negative compensatory regulation across TLRs, which suggests that members of this family display a specific type of hyper-fine gene regulation, *TLR8* was low and statistically insignificant. An increasing frequency of specific alleles has led to a deleterious impact on the regulated immune response, resulting in more COVID-19 cases. Conversely, the elevated prevalence of alternative alleles provided protection against this disease's infection. The results, however, would be more instructive if the study samples were drawn from a larger range of Iraqi areas and ethnic groups.

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Author Contributions

Raheem M. Hussein: Organizing the investigation, compiling clinical data from samples, analyzing samples, and writing the report. Planning, supervising, statistical analysis, writing and revising, and paper modification for the experiment were handled by Hula Y. Fadhil and Ali Hussein Alwan.

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Availability of data and materials

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Publication ethics

The study was carried out in conformity with moral guidelines that originated with the Helsinki Declaration. Prior to taking nasopharyngeal swabs and blood samples, each participant signed a written consent form. On September 1, 2022, the Mustansiriyah University/College of Science/Ethics Committee evaluated and approved the study protocol, subject information, and consent form. The approval was granted under reference No. Ref.:BCSMU/1221/00012M.

Consent for publication

Not applicable

Conflicts of Interest

Conflicts of interest have not been reported by the authors.

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