How to Cite:
Hussein, S. A., Mohammed, H. Q., & Mohammed, H. A. (2022). Exploring the significant role of TLR 7 gene polymorphism with severity infection of COVID-19 patients in Wasit province. *International Journal of Health Sciences*, *6*(S6), 9216–9225.
https://doi.org/10.53730/ijhs.v6nS6.12425

Exploring the significant role of TLR 7 gene polymorphism with severity infection of COVID-19 patients in Wasit province

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Abstract---Toll-like receptor 7 (TLR7 genes was involved in the host immune response against viral infections including SARS-COV-2. This study aimed to investigate the association between the TLR7 (rs179008) polymorphisms with the prognosis and susceptibility to COVID-19 pneumonia accompanying SARSCOV-2 infection. This case-control study included 120 individuals: 80 COVID-19 patients (severe and mild) and 40 controls. Polymorphisms (TLR7 rs179008) were genotyped by Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea after sent PCR products. This study also investigated predictors of mortality in COVID-19 severity through logistic regression. The mutant'T/T' genotypes and the 'T' alleles of TLR7 (rs179008) polymorphisms were significantly associated with increased risk of COVID-19 severity. Our study illustrated that the males harbouring the TT genotype of TLR7 rs 170008 polymorphism could be more susceptible to COVID-19 pneumonia than females having the same genotype.

Keywords---COVID-19, risk factors, sanger sequencing, toll-like receptors, gene polymorphism.

Introduction

In December 2019, a novel infectious disease, called COVID-19 pneumonia, emerged in Wuhan City, China, and is spreading globally. Unfortunately, the ultimate fate of the COVID-19 pneumonia outbreak is unknown, as the situation is rapidly evolving [1]. The innate immune system provides the first-line protection against invading organisms and it is potentially important in SARS-CoV-2 infection [2]. The innate immunity includes a family of receptor proteins, named pattern recognition receptors (PRRs), of which, toll-like receptors (TLRs) are important members. TLRs can identify pathogen associated molecular patterns (PAMP) and respond by eliciting inflammatory responses to eliminate the invading organisms [3]. TLR signaling has a pivotal role in the regulation of cytokine expression; hence TLR signaling could be crucially implicated in cytokine storm of SARS-CoV-2 infection [4]. Furthermore, an interaction of SARS-CoV-2 spike glycoprotein with the cell surface TLRs was found which could modify the existing knowledge of COVID-19 immunobiology [5]. TLR7, provide anti-viral immunity via the identification of viral single-strand ed RNA (ss-RNA) including SARS-CoV-2 [7]. Moreover, the activation of TLR7 results in the release of pro-inflammatory cytokines and chemokines, like IFN-alpha, IFN-beta and IFN-lambda, which have been shown to aid in viral clearance and reduced replication ,TLR-7 gene located in chromosome X p22.2 [6].

There is a growing recognition that genes, especially those regulating the host immune response might confer differential vulnerability and influence the outcomes of SARS-COV-2 infection [4, 8, 9]. It is important to identify contributing genes that can help the accurate prediction of clinical outcome and fatality from COVID-19 pneumonia and therefore allow for preventive strategies in patients with a higher risk of death. TLR genes display genetic variations and allelic polymorphisms resulting in numerous immunopathological consequences in viral infections [10]. Thus, we hypothesized that the study of TLRs immunopolymorphisms may provide important clues on the susceptibility and clinical outcomes of SARS-COV-2 infection. Our secondary endpoint will be to find other predictors of mortality among patients with COVID-19 pneumonia [10].

Materials and Methods

Study samples

Totally, 80 adult patients with age group ranging from (20 -80) year suffering from Covid-19 were included in this study. The patients were diagnosed as COVID-19 positive cases using nasopharyngeal swabs using a molecular test. Those patients were selected from Al Zahra teaching hospital and Al Karma teaching hospital in the governorate of Waist/Iraq, from November 2021 to February 2022. The name, age and gender, were collected from all patients. All inclusion criteria suggested for covid19 cases were applied. The cases are classified to sever and mild according to clinical symptoms, PO2 and CT scan. The control group include forty (40) were apparently healthy individual with age group ranging from (20 -80) year without symptoms and this was indicated by rapid test. The patient group include (44) male and (36) female, while control group include (23) male and (17) female.

Genetic studies

A total 2 ml of the study participants' peripheral blood was collected into an EDTA-containing test tube. Presto™ Mini gDNA Kit was used to extract genomic DNA from whole blood (Geneaid, Taiwan) according to the manufacturer's instructions and stored at 20°C until genotyping assay. Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of samples for downstream applications. For 1µl of DNA, 200 µl of diluted Quantifluor Dye was mixed. After 5 min incubation at room temperature, DNA concentration values were detected.

Primer preparation and optimization

The sequence of primers used in molecular analysis of this study was detailed in table (1). To examine the optimum annealing temperature of primer, the DNA template was amplified with the same primer pair, (Forward) (Reverse), at annealing temperatures of 55, 58, 60, 63 and 65°C. PCR amplifications were performed with 20 µl volumes containing 10 µl GoTaq Green Master Mix (2X); 1 µl for each primer (10 pmol); 6 µl nuclease free water and 2 µl of template DNA. PCR cycling was performed with PCR Express (Thermal Cycler, BioRad, USA) with the following temperature program: denatured at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 sec; annealing at 55, 58, 60, 63 or 65°C for 30 sec; and extension at 72°C for 30 sec. A final extension incubation of 7 min at 72°C was included, followed by 10 min incubation at 4°C to stop the reactions. The mastermix preparation was detailed in table (2) at a PCR program as shown in table (3).

Table 1
Primers used in molecular study

Primer Name	Seq.	Annealing Temp. (°C)	Product Size (bp)
TLR7-F	5'- TGTAAAACGACGGCCAGTGC CCTGACTCTACTACTATCTC -3'	60	977
TLR7-R	5'- CAGGAAACAGCTATGACGAG ATGTCTGGTATGTGGTTAAT -3'		

Table 2
PCR Component

Master mix components	Stock	Unit	Final	Unit	Volume
					1 Sample
Master Mix	2	X	1	X	12. 5
Forward primer	10	µM	2. 5	µM	1
Reverse primer	10	µM	2. 5	µM	1
Nuclease Free Water					7. 5
DNA		ng/µl		ng/µl	3
Total volume					25
Aliquot per single rxn	22	µl of Master mix per tube and add			3 µl of Template

Table 3
PCR Program

Steps	°C	m: s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria. The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel electrophoresis tank. The tank was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel. For PCR product, 5 µl was directly loaded to well. Electrical power was turned on at 100 volt/ 50 mAmp for 60 min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained band s in gel were visualized using Gel imaging system.

Sequencing

PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

Statistical Analysis

Data statistical analyses were translated into a computerized data base and were done using the statistical package for the social sciences (SPSS version 18. 0 for windows, Chicago, USA) in association with Microsoft Excel. Qualitative data were related analysis by A nova and Chi-square tests. To estimate the impact of different genotypes polymorphisms on response to severity of covid-19 disease, odds ratio (OR) and 95% confidence interval (CI) were calculated according Hardy Weinberg equilibrium (HWE) was variable between wild genotype that used as reference and both heterozygous and homozygous mutant genotypes. Statistical analysis was carried out significant at $P< 0. 05$.

Results

DNA Concentration (ng/µl)

DNA concentration was detected at a range 19-33 ng/ µl.

PCR Result

120 sample that isolate from blood to amplify 977 bp for TLR-7 gene region after electrophoresis on 1. 5% agarose gel electrophoresis stained with Ethidum bromide. M: 100 bp ladder marker, were see all the band s that used in sequence analysis (Figure 1).

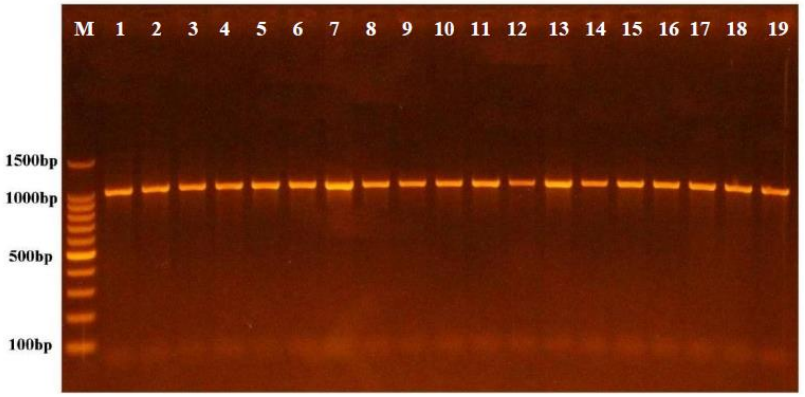

Figure 1. The amplification results of *TLR7* gene in Human samples fractionated on 1. 5% agarose gel electrophoresis stained with Eth. Br. M: 100 bp ladder marker

Sequences analysis

Relation of genotypes and allele frequency of TLR-7 "rs179008 A>T" SNP with study groups

The SNP rs179008 A>T *TLR-7* gene genotypes frequency in sever covid-19 patients describes as follows: AA (47. 5%), AT (32. 4%), and TT (32. 4%). While in mild covid-19 patients: AA (50. 00%), AT (50. 00%), and TT (0. 00%). Finally, in healthy peoples was: AA (82. 5%), AT (17. 5%), and TT (0. 00%), (Figure 2). Present study showed there was no significant difference ($p>0.05$) between genotypes (AA and AT) of *TLR-7* gene and groups (sever COVID-19 patients and mild), but there is significant difference ($p<0.05$) with TT genotype. Additionally, there was significant difference ($p<0. 05$) between genotypes (AA and TT) of *TLR-7* gene and groups (sever COVID-19 patients and control), but there was no significant difference ($p>0.05$) with AT genotype. Finally, there was significant difference ($p<0. 05$) between genotypes (AA and AT) of *TLR-7* gene and groups (healthy and mild COVID-19 patients), but there is no significant difference ($p>0. 05$) with TT genotype (Table 1).

Table 1
Genotype frequency of TLR-7 "rs179008 A>T" SNP among sever, mild covid-19 patients and control groups

A- Between sever patients and mild patients					
-008 A>T	Sever cases:#(%)	Mild cases:# (%)	OR	95% CI	P. value
AA	19 (47. 5%)	20 (50%)	0. 904	0. 376 to 2. 174	0. 823

AT	13 (32. 4%)	20 (50%)	0. 481	0. 194 to 1. 192	0. 114
TT	8 (20%)	0 (0%)	21. 189	1. 178 to 380. 924	0. 038
Total	40 (100%)	40 (100%)			
B- Between sever patients and control patients groups					
-008 A>T	Sever cases: #(%)	Control cases: # (%)	OR	95% CI	P. value
AA	19 (47. 5%)	33 (82. 5%)	0. 192	0. 069 to 0. 535	0. 0016
AT	13 (32. 4%)	7 (17. 5%)	2. 269	0. 794 to 6. 488	0. 1261
TT	8 (20%)	0 (0%)	21. 189	1. 178 to 380. 924	0. 038
Total	40 (100%)	40 (100%)			
C- Between control cases group and mild patients					
-008 A>T	Control cases: # (%)	Mild cases: # (%)	OR	95% CI	P. value
AA	33 (82. 5%)	20 (50%)	4. 714	1. 692 to 13. 131	0. 003
AT	7 (17. 5%)	20 (50%)	0. 212	0. 076 to 0. 591	0. 003
TT	0 (0%)	0 (0%)	1. 000	0. 019 to 51. 631	1. 000
Total	40 (100%)	40 (100%)			

The present study showed there is significant differences between allele frequencies of SNP rs179008 A>T *TLR-7* gene and groups (sever vs. healthy, and mild vs. healthy), where the A allele scored highest percentage in healthy, mild, and sever (92. 3%, 75%, and 63. 75% respectively) than T allele in healthy, mild, and sever (8. 7%, 25%, 29%). Finally, present study showed significant differences between allele frequencies of SNP rs179008 A>T *TLR-7* gene and groups (sever vs. healthy), (Table 2).

Table 2
Alleles frequency of TLR-7 “rs179008 A>T” SNP among sever, mild covid-19 patients and control groups

A- Between sever patients and mild patients					
-008 A>T	Sever cases: #(%)	Mild cases: # (%)	OR	95% CI	P. value
A	51 (63. 75%)	60 (75%)	0. 586	0. 297 to 1. 158	0. 124
T	29 (36. 25%)	20 (25%)			
B- Between sever patients and control groups					
-008 A>T	Sever cases: #(%)	Control cases: # (%)	OR	95% CI	P. value
A	51 (63. 75%)	73 (92. 3%)	0. 168	0. 068 to 0. 414	0. 0001
T	29 (36. 25%)	7 (8. 7%)			
C- Between control group and mild patients					
-008 A>T	Control cases: # (%)	Mild cases: # (%)	OR	95% CI	P. value
A	73 (92. 3%)	60 (75%)	3. 476	1. 377 to 8. 775	0. 0084
T	7 (8. 7%)	20 (25%)			

The present study showed the mutant TT genotype and the T allele of TLR-7 “rs179008 A>T” SNP were significantly associated with an increased risk of COVID-19 severity and disease outcome. Furthermore, our study illustrated that the males harbouring the TTgenotype of TLR7 rs170008 polymorphism could be more susceptible to COVID-19 pneumonia than females having the same genotype. This sex-dependent difference may be owing to gender-specific behaviours, genetic and hormonal factors, and sex differences related to SARS-COV-2 infection.

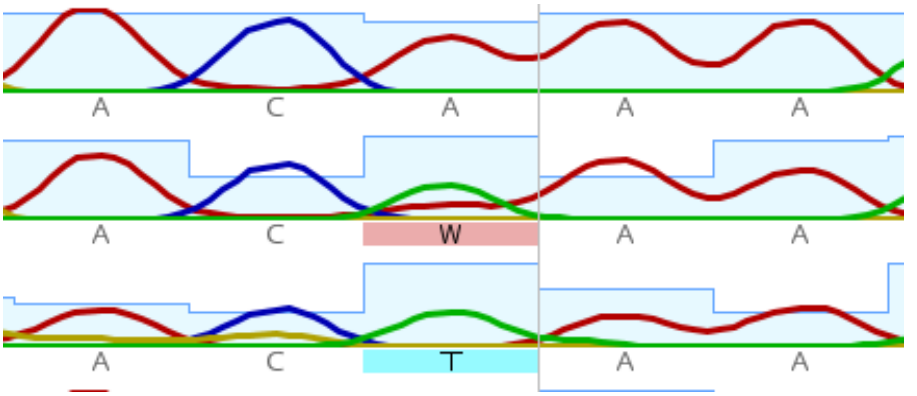

Figure 4-2. Analysis of rs179008 SNP of TLR7 gene using Sanger sequencing. Single “A” peak indicative of a A homozygous allele. Single “T” peak indicative of a T homozygous allele. Presence of the “A” and “T” peak indicative of A/T heterozygous

Discussion

TLR7 gene variations were associated with respiratory diseases. The present study is consistent with other study conducted in Egypt illustrated that the mutant ‘T/T' genotype and the ‘T' allele of TLR7 rs179008 were significantly associated with an increased risk and the susceptibility to COVID-19 pneumonia, and these results matched with present that showed high infection in patients with TT genotype and T allele of TLR7[11]. Also, TLR7 gene variations were associated with respiratory diseases. For instance, TLR7 rs179008 polymorphism showed a strong association with the development of bronchial asthma [12]. These data could be explained as the mutant ‘T' allele of TLR7 rs179008 was associated with a significant decrease in gene expression of TLR7 compared to the ‘A' allele in HCV patients and in HIV patients [13, 14]. Additionally, The substitution of A to T at the position of TLR7 rs179008 leads to an amino acid change from glutamine to leucine at position 11 of the protein-altering the TLR7 processing and cause an altered immune response [12].

Conflicting results for rs179008 were reported by different studies in Germany found that female carriers of the mutant ‘T' allele are at an increased risk of HIV-1 infection. [15]Previous study in India contrast to present study that showed the rs179008 TT genotypes were found reduced in HIV-infected individuals as compared to AA genotype was increased; their higher prevalence in health individuals clearly support that they are associated with reduced risk of acquisition of HIV infection [16]. Other study demonstrated that heterozygous

genotype 'A/T' may reduce the risk of Caucasian females to develop Chronic hepatitis B infection [17]. The genetic background of human populations can influence the susceptibility and outcome of infectious diseases. Reports suggested that the variations within the host's genome play a role in viral infection and disease progression. TLR gene variants and downstream signaling molecules could influence the ability of the affected individual to respond to TLR ligand s resulting in altered susceptibility to or course of infectious disease [16].

Strong host genetic factors that confer an increased risk to develop severe COVID-19 might serve as genomic biomarkers, along with other factors, that could be used for early diagnosis and preventative measures, and could allow the identification of possible molecular targets for treatment. In this respect, there would be a strong argument to offer hemizygous *TLR7* deficient males that have not had COVID-19 direct access to early vaccination as an effective preventative measure, similar to other patients with primary immunodeficiencies. This option has indeed been offered to the hemizygous carriers in the family of patient. Although no data is yet available to support specific management of *TLR7* deficiency or the at-risk hemizygous carriers, early hospitalization and IFN-based therapies in inborn errors of IFN signaling form rational treatment options [18]. Previous study showed the TLR7 rs179008 genotyping in male and female patients are not surprising and could be explained by the situation of the *TLR7* gene in the X chromosome which is an immune regulatory gene whose biallelic expression leading to a stronger immune response decreasing viral-load levels and inflammation in women than in men [19].

Conclusion

The mutant TT genotype and the T allele of TLR-7 "rs179008 A>T" SNP were significantly associated with an increased risk of COVID-19 severity and disease outcome, Furthermore, our study illustrated that the males more susceptible to COVID-19 pneumonia than females having the same genotype.

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