Polymorphisms of TLR 8 by Tetra- ARMS Technique Associated with COVID-19 Patients of Iraq

Zahara Ali AbdAl_Ameer and Kawther M. A. Hasan*
College of Science for Women, University of Babylon, Iraq.
***e-mail : kawtherali1972@yahoo.com**

Abstract:

The clinical spectrum of COVID-19 is extremely variable. Thus, it is likely that the heterogeneity in the genetic make-up of the host may contribute to disease severity. Toll-like receptor (TLR)8 plays a vital role in the innate immune response to SARS-CoV-2 infection.

We genotyped 60 adult COVID-19 samples taken from 20 patients, 20 recovery persons and 20 healthy people for TLR8 (rs3764880 A/G) polymorphisms usingTetra- ARMS Technique. The frequency of alleles of *TLR8* gene polymorphism of A allele is 57.5% in patients and 60.0% in control group, while frequency of G allele is 42.5% in patients and 40.0% in control group. While, the frequency of alleles of *TLR8* gene polymorphism of A allele is 55.0% in recovery and 60.0% in control group, while frequency of G allele is 45.0% in recovery and 40.0% in control group.

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beings,among those some are membrane-bound and some areendosome-specific intracellularreceptors.TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are cell surface TLRs, while TLR3, TLR7, TLR8, TLR9 are endosomal in nature (Botos*et al*., 2011;Celhar *et al*., 2012; Goulopoulou*et al*., 2016; Moreno-Eutimio*et al*., 2020).

TLRs are expressed on different immune cells, such as dendritic cells (DCs), macrophages, natural killer cells, and cells of the adaptive immunity – T cells and B cells (Angelopoulou*et al*., 2020). The TLRs can also be classified based on detecting PAMP. TLR4 detects glycoprotein, TLR7 and TLR8 detects viral single-stranded ribonucleicacid (ssRNA), TLR3 detects viral double-stranded RNA

Introduction

Toll-like receptors (TLRs) belong to the family of innate immunereceptors, which play an important role in the activation of innateimmunity, regulation of cytokine expression, indirect activation ofthe adaptive immune system, and the recognition of pathogenassociatedmolecular patterns (PAMPs)(Hedayat*et al*., 2011; Birra*et al*., 2020; Debnath*et al*., 2020).TLRs have ten family members in humans(TLR1–TLR10),and there are twelve TLRs in mice (TLR1–TLR9, TLR11–TLR13). Some of the TLRs arelocated in the cell membrane, and the others are situated in endosomes,such as TLR3, TLR7, TLR8, and TLR9 (Lester and Li, 2014).A total of 10 types of TLRs have been found in human

proposed that higher TLR7 expression among women may facilitate more efficient clearing of viral particles – though perhaps to the detriment of higher autoreactivity and increased risk of autoimmunity (Souyris*et al.,* 2019;Spiering and de Vries, 2021; Szeto*et al*., 2021)

TLR7 and TLR8 are considered phylogenetically and structurally similar, but different TLR7 and TLR8 agonists produce different types of cytokines. The elevated level of pro-inflammatory cytokines is also found to be mediated by TLR8, proved by bioinformatic analysis (Moreno-Eutimio*et al*., 2020; Fallerini*et al*., 2021).

Materials and Methods

Three ml of blood were collected 60 blood samples taken from 20 patients, 20 recovery persons and 20 healthy people. Blood injected directly in EDTA tube. Cold box was used to transfer samples for the purpose of transferring them from hospital to laboratory andsaved -20C˚ in deep freeze for using in molecular study.

The extraction and purification of DNA were performed by using FAVORGEN kit. The primer for*TLR8*rs3764880 A/G (table 1)was analyzed usingTetra- ARMS Technique. The PCR mixture for all primers used in this study was prepared according to the table (2).

(dsRNA), whileTLR9 detects viral deoxyribonucleic acid (DNA) (Choi*et al*., 2018).The TLRs play an important function in the innate immune response. Various methods including molecular, metabolic, structural, cell biology, and bioinformatics experiments have explained the exact pathways underlying TLR signaling over the last decade. TLR activation tends to be varied and active in a host of aspects of pathogen innate immune responses (Kawasaki and Kawai, 2014).

TLR7/8 are tandem duplicated genes on the X-chromosome, which are located in the endosome membrane and recognize ssRNA andsynthetic oligoribonucleotides, such as imidazoquinoline, imiquimod, and R-848. Therefore, they could be involved in the recognition ofthe SARS-CoV-2 genome (de Groot andBontrop, 2020).Additionally, genetic variation in TLR7 may be an underlying factor in observed sex biases in COVID-19 severity, where males could be predisposed to immunodeficient responses due to the location of TLR7 on the Xchromosome (Scully*et al*., 2020). TLR7 also escapes X-inactivation, generally leading to higher basal expression levels and elevated downstream TLR7-induced IFN response in women. Production of IFN-α is also increased in adult females relative to males, and it has been

Table(1): Primer used in thisstudy

Primers	Primer sequence (5' → 3')	Product size
TLR8SNP rs3764880 A/G*	Fo: AAATCACAAGTTCCCTTCTTTTCATGTA Ro: CATCACTGCATTTGATTTTCAAAATTTA Fi: GGAATGAAAAATTAGAACAACAGAACCA Ri: TTTGCTAAAGAAATAGAAGTGGCTTACAAC	423 (wild type) 272 (A allele) 209 (G allele)

***** (Hashemi-Shahri*etal*., 2014).

Table (2): Volumes of chemical materials uses in PCR assay.

Chemical materials	Volumes
Master Mix	5 µl

DNA	1-2 µl
Forward Primer	1 µl
Reveres Primer	1 µl
Deionizer D. W	18 µl
Total	≈ 25 µl

frequency and percentage. independent - sample t test used to compare between two groups,chi-square test used to detect if any relation between ordinal and nominal variable in this study. P value of ≤ 0.05 is considered significant.

Result and discussion

The frequency of genotypes and alleles of*TLR8* (rs3764880 A/G) gene polymorphism were amplified by Tetra-Amplification Refractory Mutation System (T- ARMS)technique, with tetra primers (two outer primers, and two inner primers forward and reverse). The electrophoresis result for TLR8 Tetra primer is coded as follows (423 bp = wild type, 272 bp= A allele, and 209 bp= G allele). Figure (1) show agarose gel electrophoresis of PCR products of TLR8 Tetra- primer.

The cycling conditions were as follows: initial denaturation at 95◦C for 5 min, followed by 30 cycles of denaturation at 95◦C for 30 s, annealing at 50◦C for 30 s, and extension at 72◦C for 30 s, and final extension at 72◦C for 5 min and cooling to 4◦C. Subsequently, the PCR products were detected by agarose gel electrophoresis which was visualized by staining the Ethidium bromide. The size of the PCR products of amplified DNA fragments identified by a comparison with the molecular size marker DNA (100-bp DNA ladder) and coded as following:

423 bp= wild type
272 bp= A allele
209 bp= G allele

Analysis is carried out using SPSS version 22, numerical data was expressed as mean and standard deviation, qualitative data were expressed as

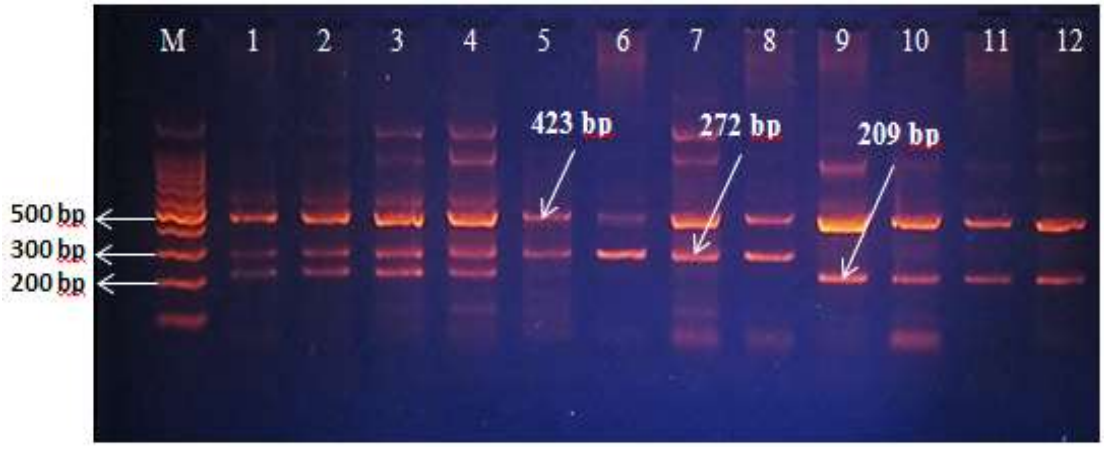

Fig. (1): Agarose gel electrophoresis of PCR products for (*TLR8*) gene polymorphism of human blood for patients and control. Lane M= molecular marker 100 bp. Line 1-4= heterozygous AG, Line 5-8 = homozygous AA and Line 9-12= homozygous GG.

The polymorphism of *TLR8*gene was done for 60 samples of blood DNA (20 patients, 20 recovery persons and 20 healthy people).When compared between patients and control,the frequency of genotypes AA, AG and GG of *TLR8* (rs3764880 A/G) gene polymorphism are respectively 40%, 35% and 25% in patients, while respectively 60%, 0% and 40% in control group. The frequency of alleles of *TLR8* gene polymorphism of A allele is 57.5% in patients and 60.0% in control group, while frequency of G allele is 42.5% in patients and 40.0% in control group (table 3).

Table (3): Genotype and allele distribution *TLR8* gene polymorphismin patients and control, shown the Odd Ratio value.

TLR8 (rs3764880 A/G) genotypes	Genotypes frequency (%)			
	Patients n=20 (%)	Control n= 20 (%)	OR (95% CI)	P- value
AA	8(40%)	12(60%)	Reference group	
AG	7(35%)	0(0%)	22.7 (1.19-432.6)	0.03
GG	5(25%)	8(40%)	0.5 (0.13- 1.9)	0.3
TLR8 alleles	Alleles frequency (%)			
A	23(57.5%)	24(60%)	0.9 (0.23-2.19)	0.8
G	17(42.5%)	16(40%)	1.11 (0.46-2.7)	0.8

and 40% in control group. The frequency of alleles of *TLR8* gene polymorphism of A allele is 55.0% in recovery and 60.0% in control group, while frequency of G allele is 45.0% in patients and 40.0% in control group (table 4).

When compared between recovery and control,the frequency of genotypes AA, AG and GG of *TLR8* (rs3764880 A/G) gene polymorphism are respectively 25%, 60% and 15% in recovering, while respectively 60%, 0%

Table (4): Genotype and allele distribution *TLR8* gene polymorphism in Recovering and control, shown the Odd Ratio value.

TLR8 (rs3764880 A/G) genotypes	Genotypes frequency (%)			
	Recovering n=20 (%)	Control n= 20 (%)	OR(95%CI)	P-value
AA	5 (25%)	12 (60%)	Reference group	
AG	12 (60%)	0 (0%)	60.29(3.2-1137)	0.006
GG	3 (15%)	8 (40%)	0.26 (0. 05-1.2)	0.08
TLR8 alleles	Alleles frequency (%)			
A	22(55%)	24(60%)	0.8(0.33 -1.9)	0.6
G	18(45%)	16(40%)	1.23((05-2.9)	0.6

12 (60%)which is morethan other two genotypes AA 5 (25%) and 3 (15%) also, in healthy control subjects was AA genotype 12 (60%) more than GG genotype 8 (40%%) and AG genotype 0 (0%), which is did not appear in control subjects. Therefore, *TLR8* (rs3764880 A/G) AG

The results for *TLR8* (rs3764880 A/G) gene polymorphism have shown that AA and AG genotype frequency 8 (40%) and 7 (35%)respectively were closed among the Covid 19 patients, which is morethan GG genotypes 5 (25%), while in recovering was AG agenotype frequency

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Turkish population and Southeast Iran respectively.

Activation of TLR pathwaysleads to secretion of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α, as well as type 1 interferon. Different TLRs, like TLR2,
TLR3, TLR4, TLR6, TLR7, TLR8, and TLR9 are potentially important in COVID-19 infection. It is also worth mentioning that we should bear in mind both the beneficial and harmful effects of TLR in confronting COVID-19 infection. TLRs could be a potential target in controlling the infection in the early stages of disease and production of vaccine against SARS-CoV-2(Khanmohammadi and Rezaei, 2021).
Manik and Singh (2022) referred to that the cytokine storm migrates into the other organ through the systemic circulation. The inflammation and the organ damage occur due to the TLR mediated NF-κB, MAPK pathway. Hence blocking these specific TLRs may alleviate the chance of SARS-COV-2 infection.Safaeiand Karimi-Googheri (2021)also revealed that women were affected by COVID-19 less than men may be through different expression of TLRs, especially TLR7 and TLR8

Conclusions

Our study found that the polymorphisms of *TLR8* (rs3764880 A/G) gene were associated with COVID-19 severity, cytokine storm, and mortality. Testing for TLR8polymorphisms may be helpful for early prediction of the course of the disease and early identification of patients who may benefit from the introduction of TLR8 antagonists in the treatment regimen.

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heterozygous genotype wassignificantly associated with increased susceptibility to Covid 19 disease.

However, the results for *TLR8* (rs3764880 A/G) gene polymorphism have shown that A allele frequency was 23 (57.5%) inpatients, 22 (55%) in recovering and 24 (60%) in control subjects, whereas G allele 17 (42.5%)in patients, 18 (45%) in recovering and16 (40%) in control subjects without significantdifference (P value = 0.8 and 0.6 respectively), as shown in Table (3 and 4).

Based on the formula of odd ratio of allele A and G: When the odd ratio of allele G (OR G)>1 implies that the allele G associated with disease. While the allele A (OR A)< 1 implies that the allele A protect against the disease. Based on our results in table (4-8 and 4-9) the odd ratio of alleles are 0.9 (95% CI = 0.23-2.19) and 0.8 (95% CI = 0.33 -1.9), that refers to the A allele protect against the Covid 19 disease.

TLRs have a critical role in pathogen recognition and activation of innate immunity and act in multiple cellular processes such as cytokine secretion, modulation of the adaptive immune response and apoptosis (Kawai *et al.,* 2007; Thada*et al.,* 2013). TLR8 is located on X chromosome and is encoded by two exons (de Groot andBontrop, 2020).TLR8 has a role in immunity against mycobacterium through IRF-7 and induced production of IFN (Cervantes *et al*., 2012).

This study is agreement with Wang *et al*., (2018), they reported genotypes frequencies at TLR8 (rs3764880 A/G) in PTB females patients and that control subjects did show significant differences in Chinese Han
population. In contrast to our findings, Dalgic*et al*., (2011) and Hashemi
et al., (2014), they reported genotypes frequencies at TLR8 (rs3764880
A/G) in PTB patients and control subjects did not show significant differences in

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