Research article

Association between SNP rs4986790 and COVID-19 infection severity among Baghdad patients

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ABSTRACT

Introduction and Aim: COVID-19, an infectious disease caused by the SARS-CoV-2 coronavirus, is distinguished by the manifestation of severe acute respiratory syndrome. Toll-like receptors (TLRs) are signaling molecules that play crucial roles in the innate immune system through their recognition of pathogen-associated molecular patterns in diverse microorganisms, including coronaviruses. The primary aim of this research was to investigate the plausible association between the TLR4 gene Asp299Gly polymorphism and the degree of infection severity among individuals who contracted COVID-19 in Baghdad, Iraq.

Materials and Methods: This cross-sectional prospective study was carried out in Baghdad to investigate the Asp299Gly polymorphism within the TLR4 gene in a cohort of 90 patients diagnosed with Covid-19. Out of the total number of patients, 45 individuals exhibited symptoms indicative of a moderate infection, while the remaining 45 patients presented with a severe illness. The Asp299Gly polymorphism was analyzed using polymerase chain reaction (PCR) technique and restriction fragment length polymorphism (RFLP).

Results: Genotyping for the Asp299Gly polymorphism showed only one among the 90 (1.1%) Covid-19 patients tested to be positive. No significant association was seen between covid-19 severity and Asp299Gly (P = 0·31) polymorphism.

Conclusion: Our study found no association between SNP rs4986790 of TLR4 gene and COVID-19 severity among Covid-19 patients in Baghdad city.

Keywords: COVID-19; SARS-CoV-2; polymorphism; Toll-like receptor 4; SNP rs4986790.

INTRODUCTION

Coronaviruses represent a class of viruses characterized by their composition of a singular RNA strand, capable of infecting a diverse range of vertebrate species (1). The identification of these pathogens in human's dates back to the 1960s (2), and they typically induce mild illnesses primarily affecting the upper respiratory tract. In the early years of the twenty-first century, a notable development occurred with the emergence of novel diseases resulting from the zoonotic transmission of highly potent beta coronavirus strains (3). These diseases were previously unrecognized. There are two significant occurrences of respiratory diseases characterized by high mortality rates, namely the Middle Eastern respiratory syndrome coronavirus (MERS-CoV) in 2012 and the initial severe acute respiratory syndrome virus (SARS-CoV-1) in 2002 (4). The emergence of a novel beta coronavirus, named SARS-CoV-2, was initially detected in the province of Hubei, China in late 2019 (5, 6). This virus exhibits resemblances to SARS-CoV-1 and has been identified as the causal agent responsible for the outbreak of the coronavirus disease in 2019 (COVID-19) (7,8)

Pathogen-associated molecular patterns (PAMPs) refer to structurally conserved patterns found in organisms. The innate immune system is able to distinguish these patterns, which are extremely important in the process of starting an early pro-inflammatory response (9). The toll-like receptor 4 (TLR-4), which serves as a significant pattern recognition receptor (PRR), is responsible for the recognition of various pathogen-associated molecular patterns (PAMPs) derived from bacteria, particularly lipopolysaccharides (LPS), viruses, and other pathogens (10). The Toll-like receptor 4 (TLR-4) has the ability to recognize specific damage-associated molecular patterns (DAMPs), which are produced by cells that have undergone lysis or cell death as a result of viral infection or injury to the host tissue. DAMPs are able to alert the immune system to the presence of potentially harmful pathogens (11).

The TLR4 gene exhibits multiple polymorphisms, among which rs4986790 is a notable co-segregating single nucleotide polymorphism (SNP) that has been extensively investigated in terms of its functional and genetic associations. At position 299, a substitution of aspartic acid (Asp) with glycine amino acid (Gly) takes place due to the presence of the rs4986790 single nucleotide polymorphism (SNP). This SNP is characterized by a transition from adenine (A) to guanine (G) at nucleotide 896 (12). The study focused on individuals diagnosed with COVID-19 from Baghdad, and their genetic material was examined to ascertain the presence of a specific genetic variation referred to as TLR-4 single nucleotide polymorphism

(SNP) Asp299Gly. Based on our research findings, a potential correlation has been identified between the aforementioned genetic mutation and the susceptibility to contracting COVID-19, as well as the potential severity of the resulting disease.

MATERIALS AND METHODS

Study design and setting

The present study included a total of ninety patients from Baghdad who were diagnosed with Covid-19 infection. Among these patients, there were 51 females and 39 males. The age range of the participants was not specified. Out of the total sample size of 90 individuals diagnosed with COVID-19, it was observed that an equal number of 45 patients exhibited mild symptoms, while the remaining 45 patients presented with severe manifestations of the infection. The presence of SARS-2-COV in the nasopharyngeal swab sample of each participant was confirmed using real-time reverse transcription-polymerase chain reaction (RT-PCR).

Ethical consideration

Samples were collected from multiple hospitals in Baghdad city, following the acquisition of official permission from the Ministry of Health and Environment. All participants provided informed consent for the collection of their personal information and the utilization of their samples in scientific research.

Blood samples and DNA isolation

A venipuncture procedure was employed to collect a 2 mL volume of blood from each participant, ensuring strict adherence to rigorous aseptic techniques. The blood samples were collected and subsequently transferred to tubes containing ethylene- diamine tetra acetic acid (EDTA) for the purpose of DNA extraction. The extracted DNA was then subjected to amplification and genotyping procedures targeting the TLR-4 gene. The technique employed for the identification of gene polymorphism was PCR-RFLP.

Determination of the TLR4 gene's genotype

PCR was employed using genomic DNA to ascertain the genotypes of TLR4 polymorphism. The amplification of the SNP rs4986790 segment of the TLR4 gene was conducted using the primers as outlined in the study conducted by Lorenz *et al*., (13). The forward and reverse primers employed in the amplification of the TLR-4 gene are provided in Table 1.

The process of DNA amplification was conducted by utilizing a combination of Master Mix (10 µl), primer (2 µl), DNA template (2 µl), and nuclease free water (6 µl). The polymerase chain reaction (PCR) was conducted utilizing a thermo cycler manufactured by a specific company in a particular country. The PCR protocol in this study involved an initial cycle at a temperature of 95°C for a duration of 5 minutes, followed by 30 subsequent cycles (14, 15). Each cycle consisted of denaturation of the primers at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The reaction was terminated by subjecting it to a final synthesis step at a temperature of 72°C for a duration of 7 minutes. The PCR products were separated using 2% agarose gel electrophoresis and subsequently stained with ethidium bromide. The PCR products underwent restriction digestion using the Nco I restriction enzyme (10 U/l; Asp299Gly; Table 2), and the TLR4 alleles were subsequently identified through 2% agarose gel electrophoresis.

Statistical analysis

The statistical analysis employed the latest edition of the widely used software SPSS for Windows. A one-way analysis of variance was conducted to examine the demographic information of patients with different polymorphisms in their copies of the TLR4 gene. The chi-square test was employed to evaluate and compare the genotype frequencies among patients exhibiting light, moderate, and severe disorders.

Table 1: The primers used in the amplification of the TLR-4 gene in this study

Gene	Primer Sequence 5`-3`
TLR4 Asp299Gly	Forward- 5′ GATTAGCATACTTAGACTACTACCTCCATG 3′ Reverse - 5′ GATCAACTTCTGAAAAAGCATTCCCAC 3′

Table 2: The restriction enzyme used and the size of the resulting restriction fragments

Gene	Polymorphism fragment	Restriction enzyme	Restriction temp °C	Length of restriction
TLR4	Asp299Gly	Nco I		Wild-type (allele A): 249 bp Asp299Gly (allele G): 223 +26 bp

RESULTS

The study included 90 COVID-19 patients, of which 51 were females and 39 males. Based on their gender these individuals were further grouped as having mild and severe infection (Table 3). Regarding patients' gender and their Covid-19 infection severity, the percentage frequency of infection was higher in females compared to male patients (Table 3). The ratio of females to males was not statistically different between the two groups (P = 0.288).

Table 3: Distribution of patients by their gender and severity of Covid-19 infection

Gender	Covid-19 infection		Total
	Mild (n= 45)	Severe (n= 45)	
	N (%)	N (%)	
Female	28 (62.22)	23 (51.11)	51
Male	17(37.77)	22(48.88)	39

Table 4: Distribution of patients by their severity of covid-19 infection and age

COVID -19 severity	No.	Age Mean ± SD
All patients	90	47.75±18.71
Mild infection	45	35.22±10.422
Severe infection	45	60.28±16.687

In a similar vein, an analysis was conducted to examine the relationship between the age of patients and the severity of their COVID-19 infection, as presented in (Table 4). Statistically significant differences were observed among the groups. The average age of patients diagnosed with severe cases of COVID-19 infection was found to be significantly higher (P= 0.014) compared to those who experienced mild infection. Specifically, the mean age of patients with mild infection was 35.22, while patients with severe infection had a mean age of 60.28.

Genotyping studies

PCR for the SNP rs4986790 segment of the TLR-4 gene yielded the expected band size of ~300 bp (Fig.1). All COVID-19 patients irrespective of mild and severe infection tested positive for this gene.

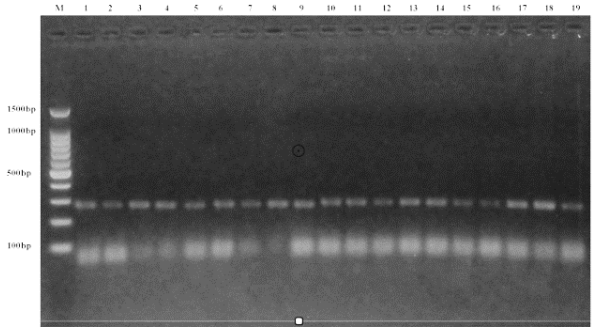

Fig.1: Representative gel picture showing amplification of TLR4 SNP rs4986790 specific gene region. M: 100bp marker. Lanes 1–19 positive for the TLR4 gene (300bp)

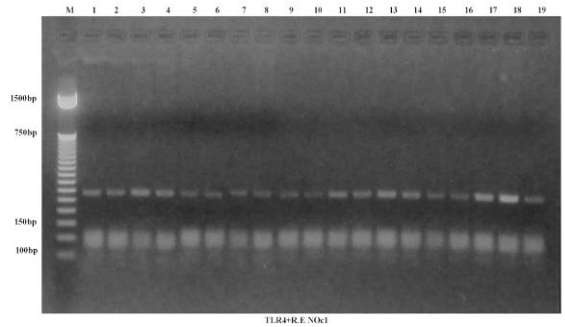

Fig.2: PCR for TLR4 SNP rs4986790 segment after digestion with restriction enzyme Nco I. M: 50 bp ladder marker. Lanes 1-19: representative samples positive for the Wild-type (allele A): 249 bp among mild Covid-19 infected patients.

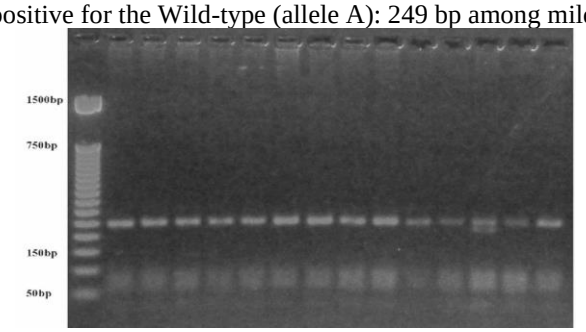

Fig. 3: PCR for TLR4 SNP rs4986790 segment after digestion with restriction enzyme Nco I. M: 50 bp ladder marker. Lanes 1-14: representative samples positive for Wild-type (allele A): 249 bp among severe Covid-19 infected patients. Lane 12: Positive for Asp299Gly (allele G): 223 +26 bp.

Restriction digestion of the TLR4 SNP rs4986790 PCR production with the restriction enzyme showed that all samples to be positive for the wild type allele A (Fig 2 and 3), except for one sample which showed the presence of allele G (Asp299Gly) genotype (Fig.3). The allele G was seen in severe covid-19 infected patient.

DISCUSSION

Toll-like receptors (TLRs), a class of proteins distributed ubiquitously in the human body, play a crucial role in virus recognition and subsequent initiation of the innate immune response, the substances in question have been found to be expressed by various human cell types, including macrophages, smooth muscle cells, endothelial cells, T cells, and dendritic cells (DC). Toll-like receptors (TLRs), which are a class of receptors, have the potential to exert a significant influence on the formulation of a vaccine targeting SARS-CoV-2, as well as in the mitigation of infection during the initial phases of the disease (13).

TLR4, a class of receptors associated with innate immunity, is situated on the cellular membrane. This component is responsible for the identification of viral proteins and other pathogen-associated molecular patterns (PAMPs). Upon detection, TLR4 triggers the synthesis of type I interferon and pro-inflammatory cytokines, which play crucial roles in the immune response against infections (16). Numerous studies have been conducted to examine the mutations occurring within the TLR4 gene and their associations with diverse diseases (11, 12). The single nucleotide polymorphism (SNP) rs4986790, located in the coding region of the TLR4 gene, is characterized by a missense mutation. This mutation replaces an aspartic acid residue with glycine at amino acid position 29. Consequently, it is probable that this alteration leads to a modified extracellular domain of the TLR4 protein (12, 13). Studies have shown the TLR4 Asp299Gly polymorphism to be associated to severe sepsis following burn injury (17), premature births, Crohn's disease (18) chronic osteomyelitis (19), increased risk of urinary tract infection in children (20) and respiratory syncytial virus in infants (21). Contrasting to these studies, reports have also suggested no correlation to exist between the Asp299Gly polymorphism with disease progression of multiple sclerosis (22) and Crohn's disease (23). Our study for TLR4 Asp299Gly polymorphism in mild and severe Covid-19 patients demonstrated that except for one patient with severe Covid-19 infection, all others to be positive for the homozygous wild type allele of SNP rs4986790. Further, no association was observed between SNP rs4986790 Asp299Gly genotype to Covid-19 phenotype among Baghdad patients. Thus we infer that SNP rs4986790 has no effect on an individual's severity to COVID-19 infection.

CONCLUSION

The present study observed a lack of statistically significant correlation between the single nucleotide polymorphism rs4986790 of the Toll-like receptor 4 gene and the severity of COVID-19 in individuals infected with the virus in Baghdad, Iraq.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Payne, S. Family Coronaviridae. Viruses. 2017: 149-158.
2. Tyrrell, D.A., Bynoe, M.L., Cultivation of a novel type of common-cold virus in organ cultures. Br Med J .1965;1(5448):1467.
3. Khan, M., Adil, S.F., Alkhathlan, H.Z., Tahir, M.N., Saif, S., Khan, M., et al., COVID-19: a global challenge with old history, epidemiology and progress so far. Molecules. 2020;26(1):39.
4. Froes, F. And now for something completely different: from 2019-nCoV and COVID-19 to 2020-nMan. Pulmonology. 2020;26(2):114-115.
5. Heymann, D.L., Shindo, N., WHO Scientific and Technical Advisory Group for Infectious Hazards. COVID-19. 2020: 395.
6. Kumar, N., Mathew, G., Huchchannavar, R. Home-based care programme (hbcp) for patients with covid-19: boon or burden during pandemic management?. Biomedicine. 2023 May 4;43(02):649-654.
7. Mamatha, S. D., Priya, S. A., Shashikala, L., Shivakumar, K. M., Chandrashekarappa, S. M. Assessment of the cognitive status of covid-19 patients and its correlation to blood pressure. Biomedicine. 2023 jul 20;43(3):1008-1012.
8. Wu, F., Zhao, S., Yu, B., Chen, Y., Wang, W., Song, Z-G., *et al.*, A new coronavirus associated with human respiratory disease in China. Nature. 2020;579 (7798):265-269.
9. Rasmussen, S.B., Reinert, L.S., Paludan, S.R. Innate recognition of intracellular pathogens: detection and activation of the first line of defense. Apmis. 2009;117(5-6):323-337.
10. Kawai, T., Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373-384.
11. Miller, R.E., Belmadani, A., Ishihara, S., Tran, P.B., Ren, D., Miller, R.J., *et al*., Damage-associated molecular patterns generated in osteoarthritis directly excite murine nociceptive neurons through Toll-like receptor 4. Arthritis Rheumatol. 2015;67(11):2933-2943.
12. Arbour, N.C., Lorenz, E., Schutte, B.C., Zabner, J., Kline, J.N., Jones, M., et al., TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000;25(2):187-191.
13. Lorenz, E., Frees, K.L., Schwartz, D.A. Determination of the TLR4 genotype using allele-specific PCR. Biotechniques. 2001;31(1):22-24.
14. Rasoul, L.M., Nsaif, M.M., Al-Tameemi, M.T., Al-Rubaii, B.A.L. Estimation of primer efficiency in multiplex PCR for detecting SARS-Cov-2 variants. Bionatura, 2022, 7 (3), 48.
15. Abbas, M.S., Ahmed, A.G., Ali, S.Q., AL-Rubaii, B.A.L. Immunological inflammatory factors in patients diagnosed with COVID-19. Biomedicine, 2023; 43(1):230-235.
16. Lorenz, E., Hallman, M., Marttila, R., Haataja, R., Schwartz, D.A. Association between the Asp299Gly polymorphisms in the Toll-like receptor 4 and premature births in the Finnish population. Pediatr Res. 2002;52(3):373-376.
17. Browning, B.L., Huebner, C., Petermann, I., Gearry, R.B., Barclay, M.L., Shelling, A.N., *et al*., Has toll-like receptor 4 been prematurely dismissed as an inflammatory bowel disease gene? Association study combined with meta-

analysis shows strong evidence for association. Off J Am. Coll. Gastroenterol ACG. 2007;102(11):2504-2512.

18. Montes, A.H., Asensi, V., Alvarez, V., Valle, E., Ocana, M.G., Meana, A., *et al.*, The Toll-like receptor 4 (Asp299Gly) polymorphism is a risk factor for Gram-negative and haematogenous osteomyelitis. Clin Exp Immunol. 2006; 143(3):404-413.
19. Karoly, E., Fekete, A., Banki, N.F., Szebeni, B., Vannay, A., Szabo, A.J., *et al.*, Heat shock protein 72 (HSPA1B) gene polymorphism and Toll-like receptor (TLR) 4 mutation are associated with increased risk of urinary tract infection in children. Pediatr Res. 2007;61(3):371-374.
20. Tulic, M.K., Hurrelbrink, R.J., Prêle, C.M., Laing, I.A., Upham, J.W., Le Souef, P. *et al*., TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide. J Immunol. 2007;179(1):132-140.
21. Reindl, M., Lutterotti, A., Ingram, J., Schanda, K., Gassner, C., Deisenhammer, F., *et al.*, Mutations in the gene for toll-like receptor 4 and multiple sclerosis. Tissue Antigens. 2003; 61(1):85-88.
22. Kroner, A., Vogel, F., Kolb-Mäurer, A., Kruse, N., Toyka, K. V., Hemmer, B., *et al.*, Impact of the Asp299Gly polymorphism in the toll-like receptor 4 (tlr-4) gene on disease course of multiple sclerosis. J Neuroimmunol. 2005;165(1–2): 161-165.
23. Lakatos, P.L., Lakatos, L., Szalay, F., Willheim-Polli, C., Osterreicher, C., Tulassay, Z., et al., Toll-like receptor 4 and NOD2/CARD15 mutations in Hungarian patients with Crohn's disease: phenotype-genotype correlations. World J Gastroenterol WJG. 2005;11(10):1489.