DOI: https://doi.org/10.54393/pjhs.v6i4.2929

PAKISTAN JOURNAL OF HEALTH SCIENCES
(LAHORE)
https://thejas.com.pk/index.php/pjhs
ISSN (E): 2790-9352, (P): 2790-9344
Volume 6, Issue 04 (April 2025)

Original Article

OPEN ACCESS

Association of TLR7 rs864058 Genotypic Variation and mRNA Expression with COVID-19 Severity and Clinical Outcomes

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ARTICLE INFO

Keywords:
Genetic Polymorphism, COVID-19, Toll-like Receptor 7, SARS-CoV-2

How to Cite:
Khalid, B., Baig, S., Angelo, S., Riaz, M., Rathore, P., & Perveen, F. (2025). Association of TLR7 rs864058 Genotypic Variation and mRNA Expression with COVID-19 Severity and Clinical Outcomes: TLR7 rs864058 Genotypic Variation and mRNA Expression with COVID-19 . Pakistan Journal of Health Sciences, 6(4), 23-29. https://doi.org/10.54393/pjhs.v6i4.2929

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Received date: 5th March, 2025
Revised date: 14th April, 2025
Acceptance date: 20th April, 2025
Published date: 30th April, 2025

ABSTRACT

Genetic variations in TLR7 could modulate the severity of COVID-19. **Objectives:** To find the association between TLR7 mRNA expression, genotypic variations, and disease severity in COVID-19 patients. **Methods:** A cross-sectional observational study was conducted on 59 PCR-confirmed COVID-19 patients at Ziauddin Hospital, Karachi (June 2022–May 2023). Blood samples were analyzed for TLR7 genotyping via PCR and Sanger sequencing. Disease severity was classified into three groups based on clinical guidelines. TLR7 mRNA expression was quantified using real-time PCR (qPCR), and hospitalization, ICU admission, and complications were documented. **Results:** Higher TLR7 mRNA expression was observed in patients with the GG genotype compared to those with the GA genotype (p=0.04). Individuals carrying the GG genotype exhibited greater hospitalization rates (75.5% vs. 40%, p=0.009), increased ICU admission (28.6% vs. 10%, p=0.041), and a higher need for mechanical ventilation (20.4% vs. 10%, p=0.049). Respiratory failure occurred more often in the GG genotype group (20.4% vs. 10%, p=0.038). **Conclusions:** It was concluded that the GG genotype of TLR7 was related to higher susceptibility to severe COVID-19 outcomes, with higher hospitalization rates, ICU admission, and respiratory failure.

INTRODUCTION

The WHO declared COVID-19 a worldwide pandemic on March 11, 2020, as countries began reporting cases globally.The COVID-19 infection continues to impact worldwide, with over 700 million COVID-19 cases and above 6 million deaths with the infection as of October 2023 [1]. Scientists first discovered enveloped RNA viruses called coronaviruses in 1968, naming them for their solar corona-like appearance under electron microscopy.The virus causes a range of symptoms collectively referred to as COVID-19, varying from mild to severe [2].Approximately 80% of patients had mild to moderate symptoms; however, some individuals develop severe conditions requiring hospitalization, intensive care unit (ICU) admission, and, in some cases, mechanical ventilation [3]. The considerable variation in disease severity has prompted extensive research to identify host genetic determinants that influence immune responses and clinical outcome [4]. The Toll-like Receptor 7 (TLR7) protein exists as a gene product located at Xp22.3 on the X chromosome short arm. The TLR7 protein appears in innate immune cells and exists on endosomal membranes to detect both single-stranded RNA (ssRNA) and synthetic oligoribonucleotides.TLR7

DOI: https://doi.org/10.54393/pjhs.v6i4.2929

plays a key role in detecting ssRNA viruses, including SARS-CoV-2, and initiating an innate immune response [5]. The pattern recognition receptor function of TLR7 transmits signal sequences to activate pathways for inflammatory cytokine production and type 1 interferon synthesis, along with antiviral immunity development [5, 6]. The TLR7 gene contains a single-nucleotide polymorphism (SNP) that modifies the TLR7 receptor structure and causes reduced immune response capacity. TLR7 exists in various immune cells like dendritic cells and B cells, and macrophages, where they detect viral RNA inside endosomal compartments. SARS-CoV-2 releases its ssRNA genome during host cell infection, where TLR7 detects the genome. The pathogen recognition by TLR7 initiates MyD88-dependent signaling, which leads to interferon (IFN-α and IFN-β) production. Interferons produced through this process establish their crucial role by both blocking viral replication and eliminating cells infected by pathogens [7]. Research has found a strong link between specific genetic variations in TLR7, particularly the 'T/T' genotypes and the 'T' allele at the rs179008 location and an increased threat of severe-type Covid-19 pneumonia [8]. On the other hand, another study revealed that individuals with the GG genotype of the TLR7 rs3853839 gene face a higher genetic risk for COVID-19 infection, severity of the disease and poorer outcomes [9]. The rs11385942 variant on chromosome 3p21.31(G/GA) significantly increases the risk of respiratory failure in COVID-19 individuals, with a 1.77-fold higher risk [10]. TLR7 polymorphisms contribute to genetic variability in immune responses, potentially leading to different immune-related consequences. These variations may increase susceptibility to RNA virus infections [11]. Genetic differences in the TLR7 gene can influence its function and expression, potentially affecting COVID-19 severity. Rare mutations that impair TLR7 activity have been tied to more severe cases, particularly in young men [12]. The role of TLR7 genetic variations in modulating immune responses and disease outcomes highlights the requirements for further research into their impact on COVID-19 severity [13].

This study aims to investigate the relationship between TLR7 mRNA expression and clinical outputs in COVID-19 patients, with a particular focus on genotypic differences and disease severity.

METHODS

This was a cross-sectional research performed on 59 COVID-19 confirmed patients who tested positive for PCR, using a convenience sampling method. Analysis of the sample size was performed utilizing the Open Epi sample size calculator with an estimated 10% proportion of COVID-19 severity linked to TLR7 polymorphisms based on past research findings [14, 15]. A confidence level of 95% (Z=1.96) and a margin of error of 5% (d=0.05) were used. 59 subjects were enrolled from outpatient clinics, general wards, and intensive care units (ICUs) at Ziauddin Hospital, Clifton, Karachi, from June 2022 to May 2023. Participants included adult patients who tested positive for COVID-19 through PCR or were treated and followed by pulmonologists at Ziauddin Hospital Clifton and those with a documented previously of COVID-19 infection. Individuals who had received chemotherapy or radiotherapy were excluded from analysis as these treatments can have profound effects on the immune system, which might confound an analysis of TLR7 expression and association with outcomes in COVID-19. Furthermore, participants with any type of malignancy were also excluded to prevent cancer-associated immune dysregulation or treatment effects from affecting the study results. The participants, aged 20 to 80 years, were both male and female. Demographics (age, weight, BMI, socioeconomic status), laboratory tests, medical history, family history, and clinical outcomes were obtained using a detailed questionnaire. The clinical severity classification in this study was conducted by pulmonologists following the WHO guidelines. COVID-19 severity was categorized as moderate or severe based on clinical and physiological parameters. Moderate COVID-19 was well-defined by the presence of pneumonia symptoms, including fever, cough, dyspnea, or fast breathing, without signs of severe pneumonia, and a SpO_2 level of ≥90% on room air. Severe COVID-19 was defined as pneumonia accompanied by at least one of the following clinical criteria: serious breathing problems, oxygen saturation (SpO_2) under 90% on room air, or a respiratory rate more than 30 breaths every minute [16]. Additionally, pulmonologists in this research categorized patients as mild, moderate, or severe using criteria such as SpO_2 levels below 94%, PaO_2/FiO_2 ratios under 300 mm Hg, or lung infiltrates covering more than 50% of the lung fields [17]. Patients were monitored throughout the study period until their recovery, death, or Leaving Against Medical Advice (LAMA). Blood samples of 59 patients were taken and kept at 4°C, and the DNA was extracted from the whole blood through the Qiagen QIAamp DNA Mini Kit 82.The TLR7 gene was amplified through PCR using the following primers (Table 1).

Table 1: Primer Sequences for TLR7 Gene Amplification

Gene	Primer Type	Sequence (5' → 3')
TLR7	Forward Primer	TGGGCTCAAATCTTTCAGTTG
TLR7	Reverse Primer	GATCACACTTTGGCCCTTGT

The amplified products were then investigated using 1% agarose gel electrophoresis.

Sanger sequencing was performed at the Lab.Genetic Lahore, Pakistan, to determine polymorphic sites.There were 59 patients with diagnosed COVID-19 who were entered into the research and were distributed into two genotype groups (GA, n=10; GG, n=49).Clinical disease severity was categorized into mild (n=29), moderate (n=12) and severe (n=18).The extraction process of total RNA from

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venous blood utilized the Gene JET Blood RNA Purification Kit from Thermo Scientific, while purity and concentration measurements were through the Nano-Drop™ 2000 spectrophotometer. Research specimens were maintained at −80°C for later analysis procedures. The Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) performed two-step synthesis of complementary DNA (cDNA) from the sample material. Hexamer primer incubation at 65°C served as the first step of the reaction before Revert-Aid reverse transcriptase, performed at 42°C, completed the process. Research laboratories kept the synthesized cDNA at −20°C for preservation.The Sensi-FAST™ SYBR Lo-ROX Kit integrated with the 7500 Real-Time PCR System was used for quantifying TLR7 mRNA expression levels by real-time PCR (qPCR). SYBR Green dye, along with cDNA and validated primers for TLR7 and GAPDH, made up the reaction mixture. The qPCR analytical method consisted of 95°C denaturation, then 50 amplification cycles, which were followed by 72°C extension. Prosecuting qPCR data was analyzed through the 2^−ΔΔCt method against GAPDH, which served as the reference gene for normalization, while the 7500 Software version 2.0.1 handled both fluorescence detection and analysis. The study was conducted after obtaining ethical consent from the Ziauddin University Ethics Review Committee (Reference code: 5360522BKBC). Informed written consent was obtained from the participants before enrollment. The statistical evaluation was carried out using SPSS version 21.0. Continuous variables such as age and TLR7 mRNA expression were given as mean ± SD and analyzed using an independent t-test. The Shapiro-Wilk test was used to know the normality of continuous variables. Categorical variables, such as gender distribution, hospitalization, ICU admission, and clinical complications, were presented as frequencies and percentages and analyzed by the chi-square test. Logistic regression analysis was done to adjust for potential confounders, including gender and age, and to assess the independent association of genotype and TLR7 expression with clinical outcomes. Significance was established at a threshold of $p<0.05$.

RESULTS

Research participants from the GA (42.3 ± 12.5 years) and GG (41.8 ± 11.7 years) genotypes showed no statistical difference in their mean age ($p=0.94$).The participant group with the GG genotype included 80% female subjects and 20% male subjects. The distribution of males to females was 80% to 20% for the GG genotype group, while the GA group showed 40.8% female and 59.2% male participants. The evaluation revealed a meaningful distinction between the two groups based on statistical analysis ($p=0.024$). The study results demonstrated that GG genotype carriers displayed elevated TLR7 mRNA expression levels at 2.15 ± 0.43 when compared to GA genotype carriers with levels measuring 1.98 ± 0.38 ($p=0.04$). The TLR7 mRNA expression levels and the demographics of participants were analyzed (Table 2).

Table 2: Demographic and TLR7 mRNA Expression Analysis

Variables	GA (n = 10)	GG (n = 49)	GG (n = 49)	p-value
Age as Mean ± SD	40.3 ± 13.2	41.8 ± 11.7	42.0 ± 11.9	0.94
Female	8 (80%)	20 (40.8%)	28 (47.5%)	0.024*
Male	2 (20%)	29 (59.2%)	31 (52.5%)	
Genotype Frequency	10	49	59	
mRNA Expression TLR7 as Mean ± SD	1.98 ± 0.382	2.15 ± 0.43	-	0.04*

Chi-square test and an independent t-test were applied. A p-value of lower than 0.05, shown by an asterisk (*), was deemed statistically significant(*).

The research revealed a major difference in hospitalization records because 75.5% of GG carriers required hospitalization, while only 40% of GA carriers needed hospital admission ($p=0.009$). The GG infected population required ICU intervention at a rate of 28.6% while the GA group admitted just 10% into such care ($p=0.041$). A total of 20.4% of people with the GG genotype required mechanical ventilation, but only 10% with the GA carrier status needed it ($p=0.049$). The distribution pattern of mild, moderate, severe and critical illness among different genotypes remained statistically the same ($p>0.05$). The mortality rate among GG and GA groups showed similar results at 6.1% versus 10%, respectively ($p=0.619$). GA group patients faced a lower incidence of pulmonary complications with respiratory failure than patients in the GG group, according to results from statistical analysis ($p=0.038$). Other complications, such as thromboembolic events, acute kidney injury (AKI), and cardiovascular complications, showed no significant variations. The study demonstrated various clinical results and complications of COVID-19 disease(Table 3).

Table 3: Comparison of Clinical Outcomes, Disease Severity, and Complications among COVID-19 Patients with GG and GA TLR7 Genotypes

Variables	GG (n=49)	GA (n=10)	Total (n=59)	p-value
Clinical Outcomes				
Hospitalized	37 (75.5%)	4 (40.0%)	41 (69.5%)	0.009*
ICU Admission	14 (28.6%)	1 (10.0%)	15 (25.4%)	0.041*
Mechanical Ventilation	10 (20.4%)	1 (10.0%)	11 (18.6%)	0.049*
Disease Severity				
Mild	24 (49.0%)	5 (50%)	29 (49.2%)	0.578
Moderate	10 (20.4%)	2 (20.0%)	12 (20.3%)	0.964
Severe	15 (30.6%)	3 (30.0%)	18 (30.5%)	0.921
Critically ill	7 (14.3%)	1 (10.0%)	8 (13.6%)	0.782
Mortality	3 (6.1%)	1 (10.0%)	4 (6.8%)	0.619
Clinical Complications				
Pulmonary Complications (Including Respiratory Failure SpO_2 <90%)	10 (20.4%)	1 (10.0%)	11 (18.6%)	0.038*

DOI: https://doi.org/10.54393/pjhs.v6i4.2929

Thromboembolic Events	12 (24.5%)	3 (30.0%)	15 (25.4%)	0.725
Acute Kidney Injury (AKI)	6 (12.2%)	1 (10.0%)	7 (11.9%)	0.835
Cardiovascular Complications	10 (20.4%)	3 (30.0%)	13 (22.0%)	0.632

Categorical variable is presented as frequencies and percentages. Chi-square test was used. A p-value of less than 0.05 is regarded as significant and is indicated with an asterisk (*).

Logistic regression analysis was done for confounders (Table 4).

Table 4: Logistic Regression Analysis Adjusting for Confounders

Variables	β Coefficient	Standard Error	Odds Ratio (OR)	95% Confidence Interval (CI)	p-value
Male vs. Female	0.81	0.35	2.25	1.15–4.38	0.026*
Age	0.12	0.07	1.13	0.98–1.30	0.083
Genotype (GG vs. GA)	1.19	0.42	3.29	1.48–7.32	0.003*
TLR7 mRNA Expression	0.69	0.30	1.98	1.10–3.55	0.021*
Hospitalization (Yes vs. No)	0.95	0.38	2.59	1.28–5.23	0.008*
ICU Admission (Yes vs. No)	1.02	0.40	2.77	1.30–5.91	0.007*
Mechanical Ventilation (Yes vs. No)	1.31	0.46	3.71	1.50–9.19	0.004*

Logistic regression was done for identifying the influence of gender, age, and genotype on TLR7 mRNA expression and clinical outcomes. Odds ratios (OR) >1 indicate a higher likelihood of the outcome occurring in the specified group. A p-value of less than 0.05 is regarded as significant and is indicated with an asterisk (*).

The results indicate that the association between TLR7 mRNA expression and genotype remains significant even after adjusting for gender and age. Additionally, genotype and TLR7 mRNA levels were significantly associated with hospitalization, ICU admission, and mechanical ventilation risk. This confirms that gender distribution differences (p=0.024) did not significantly influence the primary outcomes, as genotype and TLR7 expression independently correlated with clinical severity.

DISCUSSION

Host genetics may also be important determinants of infection severity and clinical outcome [18]. TLR7 polymorphisms have been linked with numerous communicable infections, emphasizing their role in immune response and disease susceptibility. Different research studies have identified significant links between TLR7 SNPs and infections such as COVID-19, Dengue [19], HIV-1 [20], and Chikungunya [21]. TLR7, a key TLR exhibiting a response to coronaviruses, has been linked to lung inflammation caused by respiratory syncytial virus (RSV). Variants in TLR7, such as TLR7 rs179008, are linked to a higher risk of developing pneumonia, but they do not influence the results of the disease [8]. Our study identified a TLR7 polymorphism at the rs864058 restriction site (GA) and its association with COVID-19 disease severity and clinical complications. Notably, this specific polymorphism has previously been linked to allergic rhinitis in Chinese populations, suggesting a broader role of TLR7 variations in immune-related conditions. This finding supporting the influence of TLR7 polymorphisms on immune response regulation [22]. The present study suggests that rs864058 may play a comparable role in modulating disease severity by influencing TLR7 expression levels. This aligns with findings that variations in TLR7 can alter innate immune signaling pathways, affecting viral clearance and inflammatory responses [8]. A study mentioned that rs179008 was risk factor for severe disease, likely because of its effectiveness on immune system signaling pathways. Specifically, the T/T genotype and T allele of rs179008 have been associated with increased severity, particularly in male patients, emphasizing the potential sex-linked influence of TLR7 variations [23]. Beyond rs179008 and rs864058, other TLR7 polymorphisms have been investigated for their role in COVID-19 severity. A study analyzing multiple TLR gene polymorphisms, including TLR7, reported varying degrees of association with disease susceptibility and severity [24]. Additionally, a study examining rs3853839 found potential links between this polymorphism and both COVID-19 severity and TLR7 mRNA expression levels [9]. Similarly, research on Egyptian patients indicated a significant relationship between TLR7 polymorphisms and disease outcomes, further emphasizing the work of innate immune signaling in determining the clinical trajectory of COVID patients [8]. Korean women found no significant relationship between rs864058 and CoV-2 infection, suggesting that its role in disease progression may be population-specific [25]. The discrepancy may be due to genetic diversity among populations, differences in sample size, or the low frequency of rs864058 in Koreans, making its impact on COVID-19 severity negligible in that cohort. In our study, the GG genotype was noticeably more widespread in COVID-19 patients compared to the GA genotype. The GG variant was present in 49 patients (59.8%), while the GA variant was present in 10 patients (12.2%) at rs864058. Furthermore, the subjects with the GG genotype displayed the peak levels of TLR7 mRNA expression, while participants with the GA genotype presented the low levels. The receptor serves a vital function in viral genomic RNA detection, subsequent to which it activates antiviral immune responses [26]. The

DOI: https://doi.org/10.54393/pjhs.v6i4.2929

higher TLR7 mRNA expression observed in the GG genotype linked to the GA genotype (p=0.04) suggests a potential regulatory impact of rs864058 on TLR7 transcription. Functionally, TLR7 is essential in innate immunity as it detects single-stranded RNA viruses and also in triggering an antiviral immune response. In viral infections like COVID-19, elevated TLR7 levels may enhance immune activation, leading to a stronger antiviral defense. However, excessive activation of TLR7 has also been linked to hyper-inflammatory responses, which can contribute to severe disease manifestations such as cytokine storm and tissue damage [9]. The increased mRNA expression in GG genotype carriers is associated with functional consequences, including excessive cytokine production and hyper inflammation. This up-regulation of TLR7 expression boosts the downstream activation of the NF-κB signaling procedure, resulting in an overproduction of pro-inflammatory cytokines like IL-6, TNF-α, and IFN-γ [27]. These cytokines play a central role in cytokine storm syndrome, a condition observed in severe cases, characterized by widespread inflammation, multi-organ failure, and poor clinical outcomes [28]. Increased transcriptional activity of the variant allele has been linked to greater NF-κB pathway activation, further amplifying inflammatory responses and potentially exacerbating disease severity [29]. The study found that individuals with the GG genotype were more likely for needing mechanical breathing, be admitted to the critical care unit, and be hospitalized. Covid with the GG genotype faced a higher possibility of pulmonary complications leading to respiratory failure, which appeared through SpO_2 levels below 90%. The GG genotype appears to increase disease severity because it results in critical respiratory distress among patients. A large-scale study analyzing 1.3 million Americans with COVID-19 demonstrated that people with pre-existing medical conditions endured greater probabilities of requiring hospitalization and ending in intensive care as well as death compared to those without illness histories. Existing genetic elements and pre-existing health concerns play together to form COVID-19 disease [30, 31].TLR7 mRNA was highly expressed in COVID-19 patients, particularly in the GG homozygotes and the patients with more severe disease. In addition, in patients with the GG genotype, the rates of hospitalization were higher (74%), respiratory failure (61.8%), thrombo-embolic events (26.3%), and cardiac events (22.4%), respectively (p-value<0.001). According to Klok *et al.*, thrombotic complications are frequent in severely ill COVID-19, particularly in the ICU [32]. Additionally, severely ill COVID-19 patients required ICU admission and mechanical ventilation at a significantly higher rate [33]. Numerous studies are being conducted on the TLR pathways, which may result in the creation of a new medication or vaccine to cure the illness [34, 35].

CONCLUSIONS

It was concluded that genetic variations in *TLR7* may influence COVID-19 outcomes.The GG genotype showed higher TLR7 mRNA expression and was associated with a higher risk of hospitalization, ICU admission, and mechanical ventilation compared to the GA genotype. Additionally, pulmonary complications, including respiratory failure, were less frequent in GA carriers. However, no significant differences were found in terms of overall disease severity or mortality across the genotypes. These findings suggest that TLR7 may play a significant role in the immune response to COVID-19, emphasizing the importance of further research to explore its impact on disease progression and potential therapeutic approaches.

Authors Contribution

Conceptualization: BK, SB
Methodology: BK, SA, MR, PR
Formal analysis: BK, SB, MR, PR
Writing review and editing: SB, FP
All authors have read and agreed to the published version of the manuscript

Conflicts of Interest

All the authors declare no conflict of interest.

Source of Funding

The author received no financial support for the research, authorship and/or publication of this article.

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