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Comparison of COVID-19 characteristics in Egyptian patients according to their Toll-Like Receptor-4 (Asp299Gly) polymorphism

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SUMMARY

Background: Toll-like receptor (TLR)-4 plays a vital role in recognizing viral particles, activating the innate immune system, and producing pro-inflammatory cytokines. Objectives: This cross-sectional study aimed to compare COVID-19 severity, progression, and fate according to TLR-4 (Asp299Gly) polymorphism in Egyptian patients. Methods: A total of 145 COVID-19 patients were included in this study. TLR-4 (Asp299Gly) genotyping was done using the PCR restriction fragment length polymorphism (PCR-RFLP) approach. Results: The most commonly encountered TLR-4 genotype in relation to the amino acid at position 299 was the wild-type AA (73.1%); meanwhile, the homozygous mutant GG genotype (8.3%) was the least encountered. At hospital admission, 85.8% of the AA group had free (with no ground glass opacities) chest computed tomography (CT) examination, and 16.0% were asymptomatic. On the other hand, of the AG and GG groups, 81.5% and 83.3%, respectively showed bilateral ground-glass opacities in chest CT, as well as 25.9% and 75.0%, respectively were dyspneic. Values of the total leucocytic count, C-reactive protein (CRP), ferritin, and D dimer increased in the AA<AG<GG sequence. In contrast, hemoglobin values and the absolute lymphocyte counts decreased in the AA>AG>GG sequence. ICU admission (83.3%) and in-hospital death (33.3%) rates were significantly higher in the GG group. Conclusions: In COVID-19 patients, the TLR-4 mutant G allele may be associated with a more aggressive disease course and in-hospital death. New therapeutic alternatives could be aimed at this area.

Keywords: COVID-19, Egyptian, polymorphism, toll-like receptor.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) has rapidly become a pandemic, causing many deaths worldwide [1]. Most COVID-19 patients are asymptomatic or have a mild disease, and about 10-20% are severe suffering from dyspnea, acute respiratory distress syndrome (ARDS), and multi-organ failure, necessitating hospitalization and intensive care unit (ICU) admission [2, 3]. The cause of the severe disease is thought to be excessive inflammation mediated by cytokines, chemokines, and oxygen-free radicals in a process called the cytokine storm, which ends in alveolar

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damage and increased permeability of lung endothelial cells [4].

The innate immune system, the first line of defense, recognizes various pathogens via specific pattern recognition receptors (PRRs) [5]. Toll-like receptors (TLRs) are one of the PRRs that can identify different pathogens via unique surface molecules that are shared widely between microbes and are called pathogen-associated molecular patterns (PAMPs) [6]. There are ten members of TLRs recognized in humans [7].

Many studies have linked TLR-4 with severe COVID-19 because TLR-4 is a potent stimulator of cytokine release, mainly interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α), leading to cytokine storm and ARDS [8, 9]. Moreover, the inactivation of TLR-4 was found to be of great benefit in reducing the incidence of lung injury in the murine model of sepsis [10,11]. Many studies also had suggested that mutations in TLR-4 genes can alter TLR-4 response to infection, increasing the production of pro-inflammatory cytokines [9, 12].

The purpose of this study was to discover how TLR-4 (Asp299Gly) genetic variations can affect COVID-19 clinical and laboratory features and outcomes in Egyptian patients.

PATIENTS AND METHODS

Ethical considerations

This study followed the principles outlined in the World Medical Association's Declaration of Helsinki. The Research Ethics Committee of Ain Shams University, FWA 00017585, approved the study process. After being notified about the study's purpose and protocol, each participant gave their written informed consent.

Study settings and subjects

This cross-sectional study included 145 confirmed COVID-19 adult patients by real-time reverse transcription-polymerase-chain reaction (RT-PCR). The study included all patients who visited or were admitted to Ain Shams University Isolation Hospitals, Cairo, Egypt, between January to April 2021. Patients with associated comorbid conditions like diabetes, heart, kidney, liver and lung diseases, and obesity were excluded from the study. Diagnosis and stratification of the included patients followed the World Health Organization (WHO) interim guidance [13]. All the included patients were followed until their discharge from the hospital or in-hospital death.

All participants were subjected to a careful medical history taking, focusing on age, gender, comorbid conditions, and clinical symptoms at the time of hospital admission. Patients also underwent chest computed tomography (CT) examination.

Baseline laboratory investigations, including Complete blood count (CBC) with differential analysis, C-reactive protein (CRP), ferritin, and D-dimer, were performed at The Central Laboratories of Ain Shams University Hospitals. From each participant on their admission, 4 mL venous blood was collected and divided into two vacutainer tubes: an EDTA-containing tube that was used to perform the CBC analysis by Coulter LH 780 Analyzer (Beckman Coulter, USA), then was stored at-80°C until assessing of TLR-4 (Asp299Gly) polymorphism by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and a plain tube with no additives from which serum was collected, after complete blood clotting and centrifugation at 3500 ×g for 15 minutes. The separated sera were immediately used to assess the routine

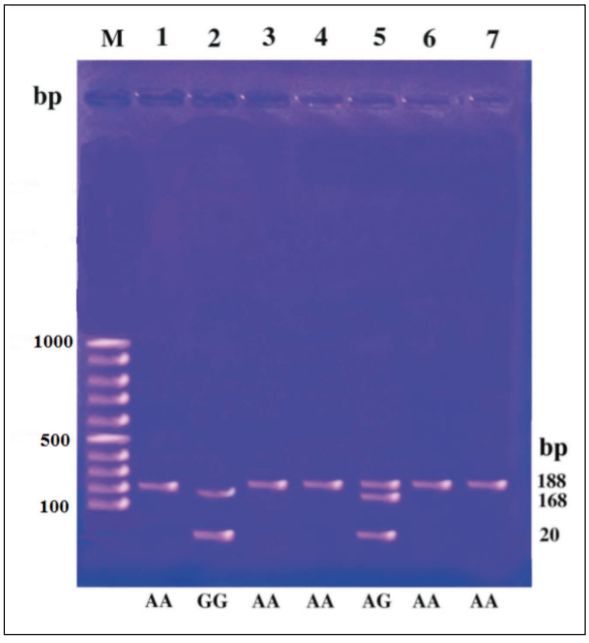

Figure 1 - Agarose gel electrophoresis showing TLR-4 (Asp299Gly) genotyping by PCR -RFLP. Lane M: DNA ladder (100-1000bp); Lane 1: the wild AA genotype at 188 bp; Lane 2: the homozygous mutant GG genotype at168 and 20 bp; Lane 3: the heterozygous mutant AG genotype at188, 168 and 20 bp.

biochemical parameters using the AU680 Analyzer (Beckman Coulter, USA).

TLR-4 Asp299Gly genotyping

The PCR-RFLP technique was used for genotyping the TLR-4 Asp299Gly (rs4986790). Human DNA amplification from EDTA whole blood was performed using a PCR kit (pub no. MAN0012900 Thermo Scientific™, USA) with human TLR-4 gene primers [14]: (forward primer 5'dAGCATACTTAGACTACTACCTCCAT 3'; Reverse primer: 5'-d GAGAGATTTGAGTTTCAATGTGGG-3'), according to the manufacturer's instructions. Then a 3 minutes centrifugation at 1000 ×g was performed to collect the supernatant for analysis. The TLR-4 gene (188bp) PCR product was then treated with Fast Digest Nco1 (Nocardia Corallina) restriction enzyme (Thermo Scientific™, USA) as directed by the manufacturer. And the digested products were electrophoresed in a 2.5% agarose gel. The product was visualized by Ethidium Bromide using an Ultra-Violet Transilluminator (Syngene, Frederick, USA): Uncut bands at 188 bp indicated the wild AA genotype of TLR-4. In comparison, bands at 168 and 20 bp indicated the mutant GG genotype (Figure 1). Bands at 188, 168, and 20 bp indicated the AG genotype [14].

Statistical analysis

We used the SPSS application (version 20) to examine the data. Mean and standard deviation (SD) were used for normally distributed data and median and interquartile range (IQR) for non-normally distributed data. Numbers and percentages represent qualitative data, whereas means and standard deviations or medians and interquartile ranges (IQR) explain quantitative data. The Mann-Whitney U and the Kruskal-Wallis tests were used to compare non-parametric quantitative variables. The Chi-square test was used to compare qualitative data. The significant results have a P of <0.05.

RESULTS

Of the 145 COVID-19 patients included in the study, 106 (73.1%) were of the homozygous AA wild-type (in the AA group), 27 (18.6%) were of the heterozygous AG genotype (in the AG group), and

Table 1 - Age and gender of the included COVID-19 patients according to TLR-4 (Asp299Gly) polymorphism.

Variable		*Genotype*			*P-value*
		AA	*AG*	*GG*	
		N = 106	*N = 27*	*N = 12*	
Age (years)	*Mean ± SD*	55.36 ± 10.42	53.65 ± 15.70	54.36 ± 16.42	0.533
	Range	18 – 88	21 – 85	17 – 86	
Gender	*Female*	61 (57.5%)	14 (51.8%)	7 (58.3%)	0.509
	Male	45 (42.5%)	13 (48.2%)	5 (41.7%)	

Notes: P-value >0.05: Non-significant; P-value <0.05: Significant; P-value <0.01: highly significant.

Table 2 - Clinical symptoms of the included COVID-19 patients according to TLR-4 (Asp299Gly) polymorphism.

Symptom n, (%)	*Genotype*						*P-value*
	AA		*AG*		*GG*		
	N = 106		*N = 27*		*N = 12*		
	N	*%*	*N*	*%*	*N*	*%*	
Asymptomatic	17	16.0%	0	0.0%	0	0.0%	*≤0.001*
Fever	64	60.3%	9	33.3%	1	8.3%	
Cough	5	4.7%	3	11.1%	1	8.3%	
Diarrhoea	3	2.8%	2	7.4%	0	0.0%	
Dyspnoea	14	13.2%	7	25.9%	9	75.0%	

Notes: P-value >0.05: Non-significant; P-value <0.05: Significant; P-value < 0.01: highly significant.

Table 3 - Baseline chest CT findings of the included COVID-19 patients according to TLR-4 (Asp299Gly) polymorphism.

CT- findings n, (%)	*Genotype*			*P-value*
	AA	*AG*	*GG*	
	N =106	*N = 27*	*N = 12*	
Free (no ground-glass opacities)	91 (85.8%)	2 (7.4%)	0 (0.0%)	≤0.001
Unilateral ground-glass opacities	0 (0.0%)	2 (7.4%)	2 (16.7%)	
Bilateral ground-glass opacities	15 (14.2%)	22 (81.5%)	10 (83.3%)	
Bilateral ground-glass opacities + pneumonia	0 (0.0%)	1 (3.7%)	0 (0.0%)	

Notes: P-value >0.05: Non-significant; P-value <0.05: Significant; P-value < 0.01: highly significant.

Table 4 - Baseline laboratory findings of the included COVID-19 patients according to TLR-4 (Asp299Gly) polymorphism.

Laboratory findings		*Genotype*			*P-value*
		AA	*AG*	*GG*	
		N = 106	*N = 27*	*N = 12*	
HB (gm/dl)	*Mean ± SD*	13.05 ± 1.63	12.17 ± 2.08	11.14 ± 2.38	*≤0.001*
	Range	10 – 17.1	7.7 – 16.2	6.9 – 14.4	
PLT (×10³/µl)	*Mean ± SD*	249.40 ± 83.61	214.06 ± 102.43	226.56 ± 117.32	0.110
	Range	22 – 546	8 – 531	40 – 516	
NT (×10³/µl)	*Median (IQR)*	8.2 (4.7 – 11.7)	8.1 (5.2 – 11.45)	8.7 (4.7 – 15.2)	0.875
	Range	3 – 7.9	1.2 – 17	4 – 21.6	
Lymph (×10³/µl)	*Median (IQR)*	1.78 (1.28 – 2.54)	1.17 (0.70 – 2)	1.15 (0.64 – 1.92)	*0.003*
	Range	0.45 – 4.2	0.12 – 4.96	0.28 – 7.58	
TLC (×10³/µl)	*Median (IQR)*	5.8(4.8 – 7.67)	6.9 (4.99 - 9.3)	9 (4.8 – 17)	*0.019*
	Range	2.2 – 25	2.12 – 29.7	0.8 – 27	
CRP (mg/L)	*Median (IQR)*	8 (4 – 20)	20 (9 - 51)	50 (30 - 120)	*≤0.001*
	Range	2 – 152	2 – 164	1 – 152	
Ferritin (ng/ml)	*Median (IQR)*	86 (17 - 226.5)	437 (126 – 763)	800 (400 – 1200)	*≤0.001*
	Range	1 – 1200	1 – 1200	128 – 2200	
D dimer (mg/L)	*Median (IQR)*	0.27 (0.12 - 1)	1.11 (0.3 – 2.1)	1.2 (0.78 – 3.7)	*≤0.001*
	Range	0.01 – 7	0.01 – 10	0.05 – 10	
Post Hoc analysis					
	AA vs AG		AA vs GG	AG vs GG	
HB (gm/dl)	*≤0.001*		*0.001*	*≤0.001*	
Lymph (×10³/µl)	*≤0.001*		*≤0.001*	*0.001*	
TLC (×10³/µl)	*≤0.001*		*≤0.001*	0.510	
CRP (mg/L)	*≤0.001*		*0.009*	*≤0.001*	
Ferritin (ng/ml)	*≤0.001*		*≤0.001*	*0.001*	
D dimer (mg/L)	*≤0.001*		*≤0.001*	*0.049*	

Notes: *P*-value >0.05: Non-significant; *P*-value <0.05: Significant; *P*-value < 0.01: highly significant; *CRP: C-reactive protein; HB: haemoglobin; Lymph: lymphocytes; NT.: neutrophils; PLT: platelets; TLC: total leucocytic count*.

Table 5 - Severity and fate of the included COVID-19 patients according to TLR-4 (Asp299Gly) polymorphism.

		Genotype			P-value
		AA	AG	GG	
		N = 106	N = 27	N = 12	
Severity n, (%)	Non-severe	57 (53.8%)	7 (26.0%)	1 (8.3%)	≤0.001
	Severe	49 (46.2%)	20 (74.0%)	11 (91.7%)	
Hospital stay (days)	Median (IQR)	9 (5.5 – 13)	11 (7 - 13)	8 (6 – 13)	0.312
	Range	2 – 30	2 – 51	2 – 24	
Outcome n, (%)	Died	0 (0.0%)	1 (3.7%)	4 (33.3%)	≤0.001
	Alive	106 (100.0%)	26 (96.3%)	8 (66.7%)	
ICU admission n, (%)	n, (%)	0 (0.0%)	10 (37.0%)	10 (83.3%)	≤0.001
Post Hoc analysis					
	AA vs AG		AA vs GG	AG vs GG	
Severity	≤0.001		≤0.001	0.001	
Outcome	≤0.001		0.047	≤0.001	
ICU admission	≤0.001		≤0.001	0.063	

Notes: *P*-value >0.05: Non-significant; *P*-value <0.05: Significant; *P*-value < 0.01: highly significant.

only 12 (8.3%) were of the homozygous GG mutant type (in the GG group). We found no significant difference between the studied groups regarding gender (*P*=0.509) and age (*P*=0.533) (see Table 1).

On hospital admission, 16.0% (17/106) of the AA group had no symptoms at all. The most common presenting symptoms in the AA and the AG groups were fever (60.3% (64/106) and 33.3% (9/27), respectively), then dyspnoea (13.2% (14/106) and 25.9% (7/27), respectively). On the other hand, dyspnoea was the most common presenting symptom in the GG group as 75.0% (9/12) of the patients had dyspnoea at the time of hospital admission (see Table 2).

According to chest CT findings, there were significant differences between the three studied groups (*P*≤0.001). Of the AA group, 85.8% (91/106) patients had free chest CT, and only 14.2% (15/106) showed bilateral ground-glass opacities. On the other hand, of the GG group, 83.3% (10/12) showed bilateral ground-glass opacities in their chest CT, and 16.7% (2/12) had unilateral ground-glass opacities. Bilateral ground-glass opacities were found in 81.5% (22/27) of the AG group, and only 7.4% (2/27) had free chest CT (see Table 3).

Regarding the baseline laboratory findings, there were significant differences between the three studied groups regarding haemoglobin levels (*P*≤0.001), absolute lymphocyte counts (*P*=0.003), and total leucocytic counts (*P*=0.019), as well as CRP, ferritin, and D dimer levels (*P*≤0.001). The median (IQR) values of the total leucocytic count, CRP, ferritin, and D dimer were significantly higher in the GG group and followed GG>AG>AA order. While haemoglobin values, the absolute lymphocyte count were significantly lower in the GG group and followed the order of GG<AG<AA (see Table 4).

Regarding COVID-19 severity, we observed significant differences between the three studied groups (*P*≤0.001). Of the GG group, 91.7% (11/12) had severe disease compared to74.0% (20/27) and 46.2% (49/106) in the AG and AA groups, respectively. There was no significant difference between the three studied groups regarding the length of hospital stay (*P*=0.312). However, the ICU admission (83.3%) and the in-hospital death (33.3%) rates were significantly higher in the GG group. No patient in the AA group was admitted to the ICU nor died during their hospital stay. Of the AG group, only one patient (3.7%) died during the hospital stay, and 10 patients (37.0%) needed ICU admission (see Table 5).

DISCUSSION

The growing significance of inflammation and immunology in COVID-19 pathogenesis has been

a major subject. TLR-4 is an inflammatory molecule that interacts with microbes' lipopolysaccharides and host heat shock proteins [15]. TLR-4 activation aids in eliminating pathogens; however, the produced radicals may harm the host's tissues due to the production of many interleukins, interferons, and other signaling chemicals that attract and trigger several immune cells to release reactive oxygen and nitrogen species [16].

TLR-4 polymorphisms are involved the development of various infectious disorders, including bacterial, fungal, parasitic, and viral infections (such as human immune-deficiency virus (HIV), Kaposi herpes simplex virus (HSV), and respiratory syncytial virus (RSV)), disruption of the receptor's function, and hindrance of pathogen identification. Also, TLR-4 (Asp299Gly) polymorphism has been linked to bacterial pneumonia susceptibility, probably due to weakened first-line defensive mechanisms [17]. Many studies conducted during the COVID-19 pandemic pointed to the importance of TLR-4 in mounting an effective immune response to SARS-CoV-2 [9, 18-21].

Although SARS-CoV-2, the virus causing COVID-19, is unlikely to activate TLR-4 directly, Aboudounya and colleagues in a previous study suggested that SARS-CoV-2 spike glycoprotein could bind TLR-4 and induce its excessive activation and abnormal signalling, allowing more inflammation, and reduction in pulmonary surfactants, breathing difficulties, and ARDS [18].

Other studies suggested that enhanced neutrophil myeloperoxidase and inflammasome activation in COVID-19 patients, especially those on ventilator support, might be responsible for uncontrolled TLR-4 mediated inflammation, contributing to immunopathological outcomes [22, 23].

In our study, 91.7% of the GG patients had a severe illness, compared to 74.0% and 46.2% of the AG and the AA patients, respectively, and the presence of the mutant G allele was linked to more severe symptoms, as well as worse chest CT and laboratory findings. Consistent with our findings, several studies suggested that the TLR-4 (Asp299Gly) G allele was associated with an increased risk of serious infections [24, 25].

Ghweil and coworkers found that fever, cough, and dyspnea were the most common encountered symptoms in COVID-19 [26]. In our study, fever was the most common presenting symptom in COVID-19 patients harbouring the wild A allele (AA and AG genotypes). At the same time, dyspnoea was the most common presentation in the mutant GG genotype patients. In a meta-analysis study, dyspnoea, rather than fever, was suggested to predict poorer outcomes in COVID-19 patients [27].

Lymphopenia is a reliable indicator of COVID-19 progression [25, 27]. In addition, previous studies reported significant leucocytosis [28] and anaemia [29] with severe COVID-19. In our study, more severe anaemia (that was indicated by both haemoglobin levels and symptomatically), leucocytosis, and lymphopenia were found in COVID-19 patients carrying the mutant G allele (GG>AG>AA). Following these facts, viral infection causes increased production of acute-phase reactants; in severe COVID-19 cases, CRP and ferritin levels rise dramatically; coagulation problems are common in COVID-19, with D-dimer levels being higher in the severe groups [30-32]. In our study, CRP, serum ferritin, and D-dimer levels significantly increased in the order of GG>AG>AA.

In a study of Egyptian patients with COVID-2019 pneumonia, Hafez has reported bilateral ground-glass opacities in severe COVID-19 infection [32]. In our study, most patients with the wild-type AA had free chest CT; meanwhile, most of the patients carrying the mutant G allele (GG and AG genotypes) had bilateral ground-glass opacities in their CT examination.

Finally, none of the AA genotype patients required ICU admission, and all of them have entirely recovered and been discharged from the hospital. Patients carrying the mutant G allele (GG>AG genotypes) have shown a significant need for ICU admission, with fatal outcomes.

One limitation of our study is the cost constraints that limited our ability to expand the sample size and test other TLR polymorphisms and their correlations with cytokine levels. Larger scale multi-centre studies are required to investigate the impact of TLR-4 gene polymorphisms on COVID-19 progression, which may help explore novel therapeutic options.

In conclusion, TLR-4 (Asp299Gly) polymorphism analysis in COVID-19 patients could aid in early disease progression prediction and intervention to enhance patient outcomes. The presence of the mutant G allele of TLR-4 (Asp299Gly) polymorphism in COVID-19 patients could be linked to a more aggressive disease course and fatal consequences.

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Conflict of interest
None

Authors contribution
We declare that all listed authors significantly contributed to the research design, data collection, analysis, and interpretation, creating or editing the paper. We further state that no one who qualifies for authorship has been excluded.

Data availability statement
Data will be available upon request.

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