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ORIGINAL ARTICLE

Role of Interleukin-6 and Tumor Necrosis Factor-Alpha Genes Polymorphisms in COVID-19 Progression

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ABSTRACT

Background: *IL6 and tumor necrosis factor-α (TNF-α) are important cytokines that determine the host defense mechanism, that play a pivotal role in COVID-19 progression.* ***Objectives:*** *To conclude the relation of IL6 174G/C and TNF-α G/A different genotypes with COVID-19 progression, and investigate the relation between IL6 174G/C gene polymorphism and IL6 serum level in COVID-19 patients.* ***Methodology:*** *A total of 100 patients, divided into three groups, participated in the present study. IL6 rs1800795 (174G/C gene) genotypes were investigated by Mutagenically Separated PCR (MS-PCR) technique, and TNF α rs1800629 (308 G/A gene) genotypes were investigated by RFLP- PCR.* ***Results:*** *As regards IL6 genotypes, GC genotype is thought to be related to COVID-19 severity as it was only present among the moderate and severe groups, and GG genotype was predominant among the mild group more than the moderate and severe groups (P-value <0.05). TNF-α genotypes; AA genotype may be a risky as it wasn't represented in the mild group, and was more predominant in the moderate and severe groups, also GG genotype may be protective as it was detected only in the mild group (P-value <0.05). IL6 C/G genotype was related to high IL6 serum level more than G/G genotype (P value< 0.001).* ***Conclusion:*** *IL6 and TNF-α genotypes are related to COVID-19 progression. High IL6 serum level was related to IL6 C/G genotype, and this may play a role in COVID-19 progression.*

INTRODUCTION

SARS-CoV-2 is a positive-sense single-stranded RNA virus of MW of 32kb[1,2]. With the emergence of the pandemic NCBI GenBank and the Wuhan-Hu-1, GenBank released the viral genome of SARS-CoV-2 sequence with accession number NC-045512.2, and MN908947 respectively[3,4].

The virus causes different clinical manifestations that ranges from mild conditions as fever, sore throat and cough to moderate conditions as pneumonia to severe conditions with high mortality rate. Severe infection manifestations are pneumonia, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), and the cytokine storm is linked to the severe complications and poor outcomes of COVID-19[5].

Cytokine storm is characterized with increased secretion of inflammatory cytokines leading to severe syndromes as fever, severe hypotension, ARDS, DIC and multi organ failure leading to death[7]. In ARDS, the increased production of different cytokines as IL-6 and TNF-α is associated with the disease severity[6].

There is a great association between polymorphism of different genes and the course of viral infection. IL6-174 C/C (rs1800795) and the TNF-α rs1800629 genotypes related to the severe form of respiratory syncytial virus infection and increased cytokine production[7,8].

In the present study, our goal was to assess the role of IL6 rs1800795 and TNF α rs1800629 different polymorphisms in COVID-19 severity and to assess the relation between IL6 rs1800795 polymorphism and serum IL6 level in covid-19 patients.

METHODOLOGY

Study participants:

This study was done in the Clinical Microbiology and Immunology Department, National Liver Institute and the Chest Department, Menoufia University Hospitals. The study involved 100 patients both (out patients and inpatients) diagnosed to be positive for Covid19 by RT PCR. The study included three groups according to the patients clinical manifestations. Group A: Included 30 Covid19 patients with mild disease

(symptomatic patients (fever, cough, anorexia, myalgia, and fatigue) without radiological evidence of viral pneumonia or hypoxia. Group B: Included 35 Covid19 patients with moderate disease (pneumonia signs as cough, fever, dyspnoea, tachypnea), but Sp O2≥ 90% on room air (RA). Group C: Included 35 Covid19 patients with severe disease with clinical signs of pneumonia (fever, cough, dyspnoea, tachypnea) plus the following signs: respiratory rate > 30 breaths/min; severe respiratory distress; or O2 saturation less than 90% on RA.

The study was submitted to get approval from Ethical Committee Board of National Liver Institute, Menoufia university by number NLI IRB0003413/00389/2022. An informed consent was taken from all subjects before the study commence.

Exclusion criteria:

Patients with (autoimmune diseases, organ transplant, malignancy, or HBV) or under immunosuppressants medication, and patients who have elevated level of IL6 and TNF as in sepsis were excluded.

The diagnosis of COVID- 19 was done clinically and by radiology as chest CT and by RT-PCR.

Laboratory investigations and detection of COVID-19

Complete blood count, liver and kidney function tests, C-reactive protein (CRP) (Roche diagnostics), D-dimer, ferritin, lactate dehydrogenase (LDH), and coagulation parameters were measured using routine clinical test kits (Human Diagnostics, Germany). Serum interleukin -6 (IL6) was measured by ELISA technique (R&D System, Inc., Minneapolis, MN). COVID-19 detection was done by Real-time PCR after extraction of RNA using a viral RNA extraction kit (QIAGEN, USA) and real-time PCR (Applied Biosystems-Life Technologies Corporation, CA).

DNA extraction for genotyping for IL6 rs1800795 (174G/C gene) and TNF α rs1800629 (308G/A gene)

Genomic DNA isolation from PBMCs were done using Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific) according to Manufacturers' instructions.

Molecular genotyping for detection of IL6 rs1800795 (174G/C gene) polymorphism

Genotyping for IL6 rs1800795 using Mutagenically Separated PCR (MS-PCR) technique[9].

Primers of *IL6(rs1800795):*

Forward G: 5′-GCACTTTTCCCCCTAGTTGTGTCTTGCG-3′

ForwardC:5′GACGACCTAAGCTTTACTTTTCCCCCTAGTTGTGTCTTGAC-3′

Reverse:5′-ATAAATCTTTGTTGGAGGGTGAGG-3′

PCR amplification was done using three primers purchased from Thermo Scientific, EU/Lithuania. PCR mixtures consisted of DreamTaq Green PCR Master Mix (2X) (Fermentas), 40 ng of DNA, 10 pmoles of each forward primer, and 20 pmoles of the reverse primer.

PCR products size was visualized using 2%agarose gel, 121bp (G allele) and 136(C allele) have been determined in comparison to 50bp DNA ladder.

Molecular genotyping for detection TNF α rs1800629 (308G/A gene) polymorphism

Genotyping for TNF α rs1800629 by the PCR-fragment length polymorphism (PCR-RFLP):

The primers were purchased from Thermo Scientific, EU/Lithuania, primers of TNF-α rs1800629 (308 G/A gene): Forward primer: 5'-AGGCAATAGGTTTTGAGGGGCAT-3',

Reverse primer: 5'-TCCTCCCTGCTCCGATTCCG-3'.

Cycles of the reaction: 1 cycle of 95°C for 2min; 35cycles of 95°C for 40s, 60°C for 40s, 74°C for 40s, and 1 cycle of 74°C for 5 min for final extension.

The amplified products were digested using 5 units Fast Digest NcoI restriction enzyme at 37°C for 10min. The resultant products were analyzed by electrophoresis in 2% agarose gel. TNF-α was genotyped and classified as follows: a single band at 107 bp identified AA homozygous individuals, one bands at 87 bp identified GG homozygous individuals, and two bands at 107, and 87 bp indicated a heterozygote.[10]

Statistical analysis:

Quantitative variables described as mean and median, and qualitative variables are described as n (%). For quantitative variables, Mann–Whitney U‑test or Kruskalwallis test were applied. For qualitative variables, Chi‑square test or Fisher's exact test were used as appropriate. Statistical analysis has been calculated by the SPSS‑17 software ,the significance was adjusted at 0.05level.

RESULTS

Our study included a total of 100 candidates (48 males and 52 females). They were selected and categorized into three groups. The age and sex of the studied groups are shown in table1.

The age between mild, moderate and severe groups were significantly different (44.37± 18.41, 55± 15.93& 60.9±15.89 respectively), but no statistical difference was determined between the three groups as regards the gender. The majority of medical health care workers infection was mild infection (30%); (P< 0.05). DM and hypertension were high in the moderate and severe groups more than the mild group (P<0.001). Also, chronic kidney disease and ischemic heart disease were more represented in the severe group more than the mild and moderate groups (P< 0.05) (table1).

Table 1: Demographic data and associated comorbidities of the studied patient groups

	Group A Mild (n=30)		Group B Moderate (n=35)		Group C Severe (n=35)		Test	P–value	Post-hoc
Age Mean ±SD	44.37±18.41		55.00±15.93		60.91±15.89		8.056#	0.001*	P1=0.036 P2<0.001 P3=0.425
	No.	**(%)**	**No.**	**(%)**	**No.**	**(%)**			
Sex									-------
Female	20	66.7%	16	45.7%	16	45.7%	X^2=3.693	0.157	-------
Male	10	33.3%	19	54.3%	19	54.3%			-------
Medical health worker									P1=0.052
Yes	9	30%	2	5.7%	2	5.7%	Fisher's Exact Test	0.01*	P2=0.052
No	21	70%	33	94.3%	33	94.3%			P3=1
DM									P1=0.007
Yes	1	3.3%	11	31.4%	18	51.4%	X^2 = 20.905	<0.001*	P2<0.001
No	30	100%	24	68.6%	17	48.6%			P3=0.435
Hypertension									P1=0.002
Yes	1	3.3%	13	37.1%	21	60%	X^2 = 26.152	<0.001*	P2<0.001
No	30	100%	22	62.9%	14	40%			P3=0.282
Ischemic heart disease									P1=0.729
Yes	0	0%	3	8.6%	8	22.9%	Fisher's Exact Test	0.001*	P2=0.018
No	30	100%	32	91.4%	27	77.1%			P3=0.564
Chronic kidney disease									P1=0.17
Yes	0	0%	5	14.3%	6	17.1%	Fisher's Exact Test	0.048*	P2=0.08
No	30	100%	30	85.7%	29	82.9%			P3>0.999
Liver cirrhosis									-------
Yes	0	0%	2	5.7%	3	8.6%	Fisher's Exact Test	0.369	-------
No	30	100%	33	94.3%	32	91.4%			-------

#: ANOVA test, X^2: Chi-square test P1=Group A&B, P2=Group A&C, P3=Group B& C

Lymphopenia, increased WBCs, neutrophils level, platelet count, D-dimmer, LDH and IL6 level were significantly high in the severe group more than the mild and moderate groups (P< 0.001), also ferritin and CRP were higher in the moderate and severe groups than the mild group (P< 0.001) (table2).

Table 2: Laboratory parameters of the studied patient groups

	Group A Mild (n=30)	Group B Moderate (n=35)	Group C Severe (n=35)	ANOVA test	P–value	Post-hoc
Hemoglobin%(gm/dl) Mean ±SD	12.257±1.09	11.229±2.5538	11.603±2.0115	2.131	0.124	------- ------- -------
WBCs ($\times 10^3$ /mm3) Mean ±SD	7.883±1.7335	7.889±3.8399	11.42±5.3395	8.845	<0.001	P1=1.000 P2=0.002 P3=0.001
Lymphocytes Mean ±SD	2863±938.617	1042.29±738.138	739.71±447.237	79.380	<0.001	P1<0.001 P2<0.001 P3=0.251
Neutrophils Mean ±SD	4870±758.469	5684±1164.212	7646.29±4167.13**1**	10.028	<0.001	P1=0.631 P2<0.001 P3=0.006
Platelets ($\times 10^3$ /mm3) Mean ±SD	300.20±66.755	221.54±79.27	247.94±123.555	5.749	0.004	P1=0.003 P2=0.085 P3=0.733
CRP Mean ±SD	11.67± 6.168	49.226± 27.068	69.48± 49.38	24.459	<0.001	P1<0.001 P1<0.001 P3=0.039
D- Dimer Mean ±SD	0.28± 0.09613	75.367± 255.79	995.795± 1525.46	12.427	<0.001	P1=1.000 P2<0.001 P3<0.001
Ferritin Mean ±SD	88.33± 36.76	547.47± 735.927	821.7054± 776.304	10.937	<0.001	P1=0.013 P2<0.001 P3=0.220
LDH Mean ±SD	329.7± 47.25	275.57± 78.21	460.8± 159.123	27.019	<0.001	P1=0.141 P2<0.001 P3<0.001
IL6 Mean ±SD	2.633± 0.616	11.806± 13.117	82.006± 149.73	8.044	0.001	P1=1.000 P2=0.002 P3=0.004

Regarding IL6 (rs1800795), GG genotype and G allele were more predominant in the mild group (100%) more than the other two groups (P< 0.005), while GC genotype and C allele seem to be associated with COVID-19 severity as they were only represented among the moderate (28.6%& 14.3%) and severe groups (22.9%& 11.4%) (table3).

Table 3: Comparison between the studied patient groups according to IL6 (rs1800795) gene polymorphism.

	Group A Mild (n=30)		Group B Moderate (n=35)		Group C Severe (n=35)		Test	P–value	Post-hoc
	No.	(%)	No.	(%)	No.	(%)			
GG	30	100%	25	71.4%	27	77.1%	X^2 =9.794	0.007*	P1=0.004 P2=0.017 P3>0.999
GC	0	0%	10	28.6%	8	22.9%			
CC	0	0%	0	0%	0	0%			
G allele	60	100%	60	85.7%	62	88.6%	X^2 =8.826	0.012*	P1=0.005 P2=0.022 P3>0.999
C allele	0	0%	10	14.3%	8	11.4%			

χ2 = Chi-square test

IL6 (rs1800795) polymorphism distribution displayed no significant relation with the death rate among the studied patient groups (P value> 0.05) (table4). Also, IL6 genotypes weren't related to D-dimer, LDH and CRP (P value> 0.05). But a significant relation was found between IL6 genotypes regarding ferritin (P value<0.05) (table5). Regarding the relation between IL6 genotypes and IL6 serum level, C/G genotype related to high IL6 serum level significantly(median= 21) in comparison to G/G genotype (median = 6); (P value<0.001) (figure1).

Table 4: The impact of IL6 (rs1800795) polymorphism on the outcome (survival) among the studied patient groups.

Genotype	Survival (70)		Death (30)		OR (95% CI)	P-value
	NO.	%	NO.	%		
G/G	60	85.7%	22	73.3%	1®	0.15
C/G	10	14.3%	8	26.7%	0.46 (0.16-1.31)	
G allele	130	92.86%	52	86.67%	1®	0.257
C allele	10	7.14%	8	13.33%	0.5 (0.19-1.33)	

®: Reference value for odds ratio, OR: Odds ratio, CI: Confidence interval

Table 5: The relation of IL6 (rs1800795) polymorphism with the laboratory parameters within the studied groups.

	G/G (N= 82)	C/G (n=18)	Mean difference (95% CI)	P-value
D-dimer Mean ±SD	339.01±1051	538.89 ±843.8	0 199.88 (-319.56 - 719.33)	0.45
LDH Mean ±SD	368.26 ±131	303.72 ±135.5	0 -64.53 (-131.80 - 2.73)	0.063
Ferritin Mean ±SD	395.84 ±517.5	1006.24±1099	0 610.40 (275.52 - 945.28)	<0.0001*
CRP Mean ±SD	44.04±41.3	49.62±38.7	0 5.58 (-15.25 - 26.41)	0.6

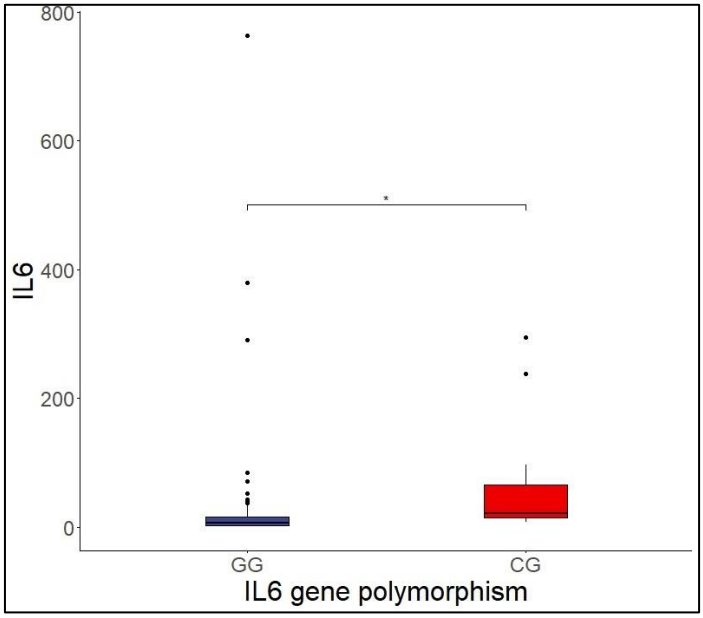

Fig. 1: The relation of IL6 (rs1800795) polymorphism with serum IL6 level within the studied groups.
There was a statistically significant difference (P value< 0.05)
U= Mann- Whitney U test was used for statistical analysis.

Regarding the TNF α (rs1800629), GG genotype is thought to be protective as it was represented in the mild group only (20%). AA genotype could be a risky as it wasn't represented in the mild group, and was predominant in the moderate and severe groups (14.3%& 17.1%). Also, G allele was more represented in the mild group (60%) than the moderate and severe groups (43%& 41.4% respectively), (table 6& figure 2).

Table 6: Comparison between the studied patient groups according to TNF α (rs1800629) gene polymorphism.

	Group A Mild (n=30)		Group B Moderate (n=35)		Group C Severe (n=35)		Test	P–value	Post-hoc
	No.	(%)	No.	(%)	No.	(%)			
GG	6	20%	0	0%	0	0%	Fisher's Exact Test	0.0005	P1=0.003 P2=0.004 P3>0.999
GA	24	80%	30	85.7%	29	82.9%			
AA	0	0%	5	14.3%	6	17.1%			
G allele	36	60%	30	43%	29	41.4%	X^2 =5.399	0.067	------- ------- -------
A allele	24	40%	40	57%	41	58.6%			

χ2 = Chi-square test

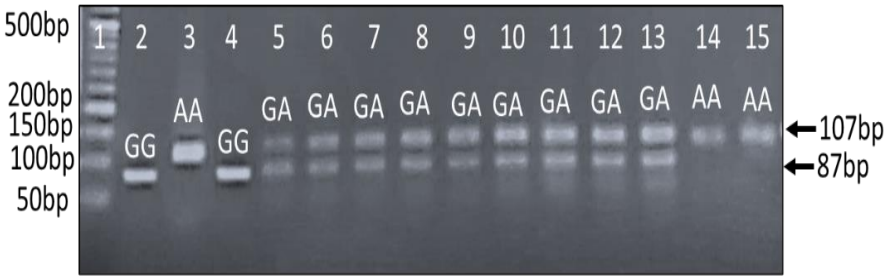

Fig. 2: Genotyping of TNF α by RFLP. Lane 1 shows 50 bp ladder, lanes 3, 14 and 15 show homozygous AA genotype yielded 1 band of 107 bp. lanes 2 and4: homozygous GG genotype yielded 1 band of 87 bp. lanes 5,6,7,8,9,10,11,12, and 13: heterozygous GA genotype yielded 2 bands of 107 bp and 87 bp.

Regarding TNF α genotypes and the mortality rate, the A allele increased the mortality rate (table7). Also, G/A and A/A genotypes related to high IL6 serum level compared to the G/G genotype (figure3).

Table 7: The impact of TNF α (rs1800629) polymorphism on the outcome (survival) among the studied patient groups

Genotype	Survival	Death	OR (95% CI)	P-value
A/A	5 (7.1%)	6 (20%)	1®	0.028*
G/A	59 (84.3%)	24 (80%)	2.95 (0.82-10.59)	
G/G	6 (8.6%)	0 (0%)	NA (0.00-NA)	
A allele	69 (49.29)	36(60)	1®	
G allele	71 (50.71)	24 (40)	1.54 (0.84-2.85)	0.216

®: Reference value for odds ratio, OR: Odds ratio, CI: Confidence interval
NA: not applicable

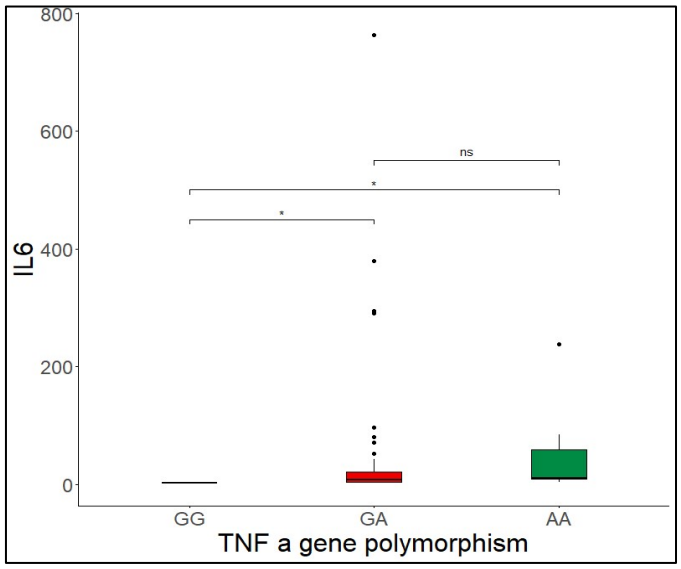

Fig. 3: The relation of TNF α (rs1800629) polymorphism with serum IL6 level within the studied groups.
There was a statistically significant difference (P value< 0.05)
Kruskal- Wallis test was used for statistical analysis.

DISCUSSION

COVID-19 infection ranges from asymptomatic cases to a multi organ failure. Host factors influence the clinical presentation and infection outcome. Effect of different human gene genotypes involved in the antiviral responses to SARS-CoV-2 has been determined, and there is evidence that some gene polymorphisms may affect COVID-19 severity[13]. IL6 genotype (rs1800795) and the TNF α rs1800629 genes polymorphisms were related to severe RSV cases and with increased cytokine production[8].

Regarding IL6 G/C genotypes in our study, all mild patients carried GG genotype and G allele (100%), while GC genotype and C allele were associated with COVID-19 severity as they were only represented among the moderate and severe groups. Similar results were previously reported in three studies[12,13,14]. On the contrary, two studies[15,16] reported that no difference in genotype or allele distribution of SNP of IL-6 gene in severe cases of COVID-19 and mild cases. This inconsistency in the results reported by different studies were explained by different sample size, inclusion and exclusion criteria of patients, geographic area, ethnicity, and racial heterogeneity[16].

No significant relation was reported between IL6 (rs1800795) genotypes or allele distribution and the mortality rate. Our results agreed with those reported by Aladawy *et al*., [15] and Batur and Hekim[5] who reported in their studies no significant relation was detected between the mortality rates, and IL6 gene polymorphisms (p> 0.05). However, Smieszek *et al.*,[17] in their study said that IL6 (rs1800795) G/C genotype was related to high mortality rate. Also, Rodrigues et al.,[18] detected a positive relation between C allele of IL6 and COVID-19 mortality rates and suggesting that C allele is involved in COVID-19 severity.

In our study, IL6 (rs1800795) polymorphism was evaluated to elucidate its responsibility in COVID-19 progression. Our results suggest that IL6 (rs1800795) polymorphism altered IL6 level, therefore influenced host immune response against COVID-19 infection. G/C genotype related to high IL6 serum level more than G/G genotype significantly; (P value< 0.001). Similar results were reported by many studies [17,18,19,20]. This was explained by that IL6 rs1800795 SNP affects IL6 level

which in turn augments the patient's immune system, and exacerbates the inflammatory responses resulting in worsening of COVID-19 manifestations, hence increased IL-6 production is measured as a hallmark of severe COVID-19[21].

Regarding TNF α (rs1800629**)** genotypes, GG genotype was present in the mild group only, AA genotype may be a risky as it wasn't represented in the mild group, and was associated with the moderate and severe groups (P< 0.05). Also, G allele was more predominant in the mild group than the moderate and severe groups. Results similar to that reported by Saleh *et al.,*[22] and Al-Mayah *et al.,*[23] who found AA and GA genotypes were related to disease severity. This was explained by that TNF-α genetic polymorphism caused TNF-α overproduction and disease progression[23].

Regarding the relation between the TNF-α genotypes and the mortality rate, we found AA genotype and A allele increased the mortality rate. Saleh *et al.,* [22] also said that AA genotype increased the mortality rate.

We reported a significant increase in IL6 level with genotypes AA & GA compared to GG genotype (P value= 0.005). This association may be explained by when cytokine storm occurred in COVID-19, it increases the production of TNF-α, interleukin 1, IL-6, IL-8, IL-12, and IFN-γ; thus, increased IL-6 and TNF-α increased COVID-19 severity, so polymorphisms in inflammatory cytokines genes affect the susceptibility and severity of COVID-19 [24].

CONCLUSION

IL6 G/C genotype and C allele were related to COVID-19 severity, while G/G genotype and G allele were related to the mild form of COVID-19. TNF *α* AA genotype was related to COVID-19 severity, and G/G genotype was related to mild form of COVID-19. IL6 G/C genotype was related to high IL6 serum level more than the G/G genotype, also TNF *α* AA& GA genotypes were related to high IL6 serum level more than GG genotype. These results suggest that the use of anti-IL6 and anti-TNF may help in preventing the disease progression. Though, further studies are needed to reveal the responsibility of anti-IL6 and anti-TNF in the disease outcome.

Declarations:
Consent for publication: Not applicable
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Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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