Frequency of interleukin-6 rs1800795 (-174G/C) and rs1800797 (-597G/A) polymorphisms in COVID-19 patients in Turkey who develop macrophage activation syndrome

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SUMMARY

SARS-CoV-2 (COVID-19) has infected over 100 million people since it appeared in Wuhan, China just 1 year ago. This study aimed to evaluate the relationship between interleukin-6 (IL-6) gene polymorphisms -174G/C and -597G/A and COVID-19 course. The study included a total of 70 patients aged 18–45 years who were hospitalized in our hospital and diagnosed with COVID-19 in Turkey between March and November 2020. Of these, 40 patients required intensive care admission due to macrophage activation syndrome (MAS) and 30 patients did not develop MAS or acute respiratory distress syndrome. The frequency of IL-6-174G/C and -597G/A polymorphisms was determined. There were statistically significant differences between the groups in terms of -174G/C allele and genotype frequency and comparison with Hardy-Weinberg distribution (χ^2=10.029, df=1, p=0.002 and χ^2=9.998, df=1, p=0.002, respectively). The frequency of the GG genotype was significantly higher in the MAS group compared to the non-MAS group (p=0.002). The G allele was also significantly more frequent in the MAS group compared to the non-MAS group (p=0.032). Analysis of the -174G/C polymorphism in patients with MAS showed that the G allele may be a risk factor for increased serum IL-6 levels and progression to MAS.

Introduction

In the year since novel coronavirus disease 2019 (COVID-19) first appeared in Wuhan, China in December 2019, there have been over 70 million confirmed infections worldwide and this number continues to rise daily. Most infected patients are asymptomatic or present with mild symptoms such as anosmia/ageusia, sore throat, malaise, and arthralgia. However, the clinical course can be more severe in older people, patients with hypertension and diabetes, patients with HIV or long-term immunosuppressive therapy, and pregnant women (1, 2).

Severe COVID-19 most frequently manifests with acute respiratory distress syndrome (ARDS) with hypoxemic respiratory failure, and macrophage activation syndrome (MAS). In both of these clinical conditions, overexpression of proinflammatory cytokines causes endothelial dysfunction and can result in vital organ injury, especially in the lungs (3). The most commonly used prognostic parameters in COVID-19 include D-dimer, ferritin, leukopenia, fibrinogen, prothrombin time, and interleukin-6 (IL-6) levels (4, 5).

Among the cytokines produced by active macrophages, IL-6 is the main cytokine contributing to the development of MAS in COVID-19. The most important evidence confirming this is the correlation between IL-6 levels and disease severity observed in studies of COVID-19 patients (6). IL-6 activates the Janus kinase system by binding to cell membrane receptors IL-6R or gp130 (7). The activated system enables naive T cells to rapidly transform into effector T lymphocytes. Cytokines synthesized from these cells play an important role in increased vascular permeability, multiorgan damage, and reduced myocardial contractility. Although the importance of risk factors such as age and comorbidities has been established, MAS can still be difficult to predict in young patients and those with no comorbidities (6). In a single nucleotide polymorphism (SNP) study conducted in patients with septic shock, which is another condition in which IL-6 levels play an important role in clinical course, individuals with the rs1800795 (-174G/C) CC genotype were found to have more severe disease (8). In another study evaluating

the relationship between plasma IL-6 concentration and -174G/C genotype levels, patients with the GG genotype showed a larger increase in IL-6 level (9).

The aim of the present study was to investigate the relationship between the frequency of IL-6 rs1800795 (-174G/C) and rs1800797 (-597G/A) polymorphisms and progressive clinical course in COVID-19 patients younger than 45 years of age with and without comorbidities.

Patients and Methods

The study included patients who presented to the emergency department of Erzurum Regional Training and Research Hospital with symptoms such as recent-onset fever, cough, shortness of breath, malaise, and sudden loss of taste and smell and had history of contact with a confirmed or suspected COVID-19 patient or travel abroad within the last 14 days.

Patients at high risk for COVID-19 admitted between March 2020 and November 2020 underwent posterior-anterior chest X-rays and those with suspicious lesions were evaluated in more detail using high-resolution thoracic computed tomography (CT). COVID-19 was diagnosed using real-time polymerase chain reaction (PCR) testing of nasopharyngeal swab samples. The study initially included 77 patients aged 18–45 years with PCR-confirmed COVID-19 and no known comorbidities who were either admitted to the intensive care unit due to a clinical picture of MAS (n=43) or treated in the COVID-19 ward due to poor general condition but did not develop MAS or ARDS (n=34). In addition to comorbidities such as chronic obstructive pulmonary disease (COPD), diabetes, uncontrolled hypertension, coronary artery disease, and malignancy, other exclusion criteria were history of infectious or inflammatory conditions or invasive surgery within the last month and high fasting blood glucose. The patients' history and laboratory results obtained while in hospital were used to evaluate the patients in terms of exclusion criteria. The cardiology, chest diseases, and internal

medicine departments were consulted to determine the presence of coronary artery disease, asthma, COPD, and diabetes. Three patients in the MAS group were excluded from the study due to abnormal fasting blood glucose levels. From the group of patients who did not develop MAS or ARDS, 1 patient with COPD and 3 patients with abnormal fasting blood glucose were excluded from the study. The patients' biochemical parameters including liver and kidney function tests, hematological parameters, coagulation parameters, ferritin, D-dimer, troponin-I, C-reactive protein (CRP), and arterial blood gas parameters were examined upon admission and updated daily.

Measurement of biochemical markers

Blood samples were collected after 15 minutes of semi-supine rest, from an antecubital vein into tubes containing ethylenediaminetetraacetic acid (EDTA) to prevent coagulation. Troponin I concentrations were measured by chemiluminescent immunoassay using an Immulite 2500 (Siemens Medical Solutions, Erlangen, Germany). IL-6 was measured by enzyme-linked immunosorbent assay (Elabscience human ELISA kit, UK).

Molecular Analyses

DNA Isolation Protocol

Blood samples were collected into EDTA tubes and DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quality of the isolated DNA was measured by Nanodrop (ND-1000, Thermo Fischer Scientific, Wilmington, DE, USA).

Analysis of rs1800795 and rs1800797 SNPs

Allele-specific SNP Type Assays were performed using a Fluidigm Flex Six™ Genotyping IFC (Fluidigm Corp., South San Francisco, CA, USA). Specific target amplification (STA) was

performed to increase the number of molecular targets at the beginning. The determined thermal cycle program was run on a Bioer Gene Pro thermal cycler (95°C for 15 min followed by 14 cycles of 95°C for 15 s and 60°C for 4 min). SNP Type Assay mixes and sample mixes were prepared according to the manufacturer's protocol. After loading a dynamic array with 4 μL of each 10× assay mix and 5 μL of each sample mix, it was placed on IFC Controller HX (Fluidigm) and the loading process was completed. The dynamic array was then placed in the BioMark system (Fluidigm), which performs the thermal cycling and fluorescent image acquisition. The build-in data collection software of the BioMark system was used. Genotyping application, ROX passive reference, and SNPtype-FAM and SNPtype-HEX probe types were selected. The SNPtype E Flex Six v1 protocol was used for thermal cycling and image capture. The genotypes of the samples were evaluated in Arı Genetic Laboratory / Erzurum.

Statistical analysis

The data were analyzed using SPSS Statistics version 24.0 for Windows (IBM Corp., Armonk, NY, USA). Comparisons of characteristics between patients with and without MAS were analyzed by chi-squared (χ^2) test for categorical variables and independent-samples t-test or Mann–Whitney U test for continuous variables, as appropriate. Differences in allele and genotype frequencies were compared between the MAS and non-MAS groups using Pearson's χ^2 test. Statistical significance of the observed genotype frequencies was evaluated according to the Hardy–Weinberg model and compared to the expected genotype frequencies. Independent-samples t test was used to compare demographic data and laboratory parameters between groups. A p-value less than 0.05 was considered statistically significant.

Definitions and Diagnosis

Fever was defined as an axillary temperature of 37.3°C or higher. Secondary bacterial infection was defined as the presence of signs and symptoms of bacteremia or pneumonia, with growth of a new pathogen demonstrated in culture of sputum or endotracheal aspirate from the lower airway. Patients diagnosed as having ventilator-associated or hospital-acquired pneumonia were treated according to available guidelines. ARDS was diagnosed and graded according to the Berlin 2015 diagnostic criteria (10). Patients with elevation in daily cardiac-specific troponin level were evaluated by echocardiography for emerging cardiac pathologies. Coagulopathy was defined as prothrombin time 3 s longer than normal and activated partial thromboplastin time 5 s longer than normal. According to the patients' disease severity, treatment was planned based on the COVID-19 diagnosis and treatment guidelines for adults issued by the Turkish Ministry of Health. Patients with signs such as refractory fever, CRP and ferritin levels that remained high or continued to rise, D-dimer elevation, cytopenia manifesting as thrombocytopenia or lymphopenia, abnormal liver function tests, hypofibrinogenemia, or elevated triglyceride levels in spite of treatment were monitored for MAS. If these parameters continued to deteriorate during follow-up with no apparent secondary bacterial infection, 400 mg tocilizumab was administered for MAS unless contraindicated. Clinical and laboratory response was evaluated after 24 hours. If an adequate response was not observed, treatment was repeated at the same dose.

Results

Mean age was 42.7 ± 8.6 years in the MAS group and 39.7 ± 12.4 years in the non-MAS group. Sex distribution was 25 (62.5%) men and 15 (37.5%) women in the MAS group, 18 (60%) men and 12 (40%) women in the non-MAS group. There were no statistically significant differences in age or sex between the groups.

Comparisons of laboratory data between patients with and without MAS are shown in Table 1. Patients who developed MAS had significantly reduced white blood cell and platelet counts (p=0.05, 0.001) and significantly higher neutrophil/leukocyte ratio (NLR), aspartate transaminase (AST), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), creatine, prothrombin time, CRP, troponin, PaO_2/FiO_2 (ratio of arterial oxygen partial pressure to fractional inspired oxygen), D-dimer, ferritin, and IL-6 level (p=0.001, 0.03, 0.001, 0.02, 0.04, 0.02, 0.001, 0.001, 0.001, 0.03, 0.001, and 0.001, respectively).

IL-6 -174G/C allele and genotype frequencies of the groups and comparison with the Hardy-Weinberg equilibrium is shown in Table 2. A statistically significant difference was observed between the non-MAS and MAS groups (χ^2=10.029, df=1, p=0.002 and χ^2=9.998, df=1, p=0.002, respectively).

Comparison of genotype frequencies in the groups is shown in Table 3. In analysis of the IL-6 -597G/A polymorphism, all patients in both the MAS and non-MAS groups were found to have the AG genotype. The frequency of the GG genotype was significantly higher in the MAS group compared to the non-MAS group (χ^2=10, df=1, p=0.002). The G allele was also significantly more frequent in the MAS group compared to the non-MAS group (χ^2=4.615, df=1, p=0.032) (Table 4).

Figure 1 shows a comparison of serum IL-6 levels by genotype. Patients with the GG genotype had significantly higher IL-6 levels than those with the GC genotype (p=0.001).

Discussion

In this study, evaluation of -597G/A and -174G/C polymorphisms and levels of IL-6, which is an important cytokine in the development of MAS, revealed no significant relationship with the -597G/A polymorphism. However, evaluation of the relationship with the -174G/C polymorphism showed that the prevalence of MAS increased in correlation with G allele frequency. In addition, serum IL-6 levels were higher in individuals with the GG genotype when compared with the GC genotype.

The novel coronavirus that causes COVID-19 was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses. SARS-CoV-2 is closely related to SARS-CoV and MERS-CoV, two other coronaviruses that caused previous epidemics with high morbidity and mortality. During the global COVID-19 pandemic, over 70 million confirmed cases and 1.6 million deaths have been officially reported to date (1).

Lymphopenia is observed in most COVID-19 patients in laboratory analyses. This suggests that SARS-CoV-2, like SARS-CoV, may predominantly affect lymphocytes, particularly T lymphocytes (11). Virus particles that spread from the respiratory mucosa infect other cells, triggering a cytokine storm. Damage to T lymphocytes is believed to have an important role in cytokine storm development. Therefore, lymphopenia may be a reference parameter that can be used in the diagnosis of COVID-19 (12). Several proinflammatory cytokines are released during a cytokine storm, particularly TNF-alpha, IL-1, IL-2, IL-4, IL-6, and nitric oxide. Cytokine discharge is also the main cause of fatal complications of COVID-19 (13, 14). Treatments such as stem cell therapy for inflammatory cytokine blockade and convalescent plasma transfusion therapy also aimed mainly to suppress IL-6, IL-1, and interferon-gamma release (15, 16).

Cytokines, especially IL-6, can disrupt tissue perfusion and cause endothelial damage and microthrombus formation by increasing vascular permeability. This increase in vascular permeability causes fluid to accumulate in the lung tissue and interstitial spaces, which manifests clinically as acute respiratory failure. Favorable results have been reported on the use of the IL-6 receptor antagonist tocilizumab to prevent these complications (17, 18).

IL-6 affects various tissues and organs, especially the lungs, due to vascular and endothelial damage. Studies of SNPs of the IL-6 gene have demonstrated a strong link between the -174G/C polymorphism and the development of coronary artery disease and type 2 diabetes mellitus, with the C allele associated with increased risk for both diseases (19, 20). Another study conducted on rheumatoid arthritis patients under treatment with IL-6 antagonists suggested that the GG genotype could be protective in terms of rheumatoid arthritis, while the CC genotype may be a predisposing factor (21). In another study evaluating the -174G/C polymorphism and plasma IL-6 concentrations in obese women undergoing dietary intervention, IL-6 levels at the beginning of treatment were significantly lower in women with the CC genotype than in those with the GG genotype (9). Compared to -174G/C, there has been relatively less research on the IL-6 -597G/A SNP. One study yielded findings suggesting that it may be a risk factor for adult-onset asthma (22). However, in other studies in patients with coronary artery disease, type 2 diabetes mellitus, and systemic lupus erythematosus, no significant difference was detected related to disease development or severity (23, 24).

In the present study, we observed that parameters associated with poor prognosis such as ferritin, LDH, CRP, IL-6, and D-dimer were significantly higher in COVID-19 patients who developed MAS compared to those in the non-MAS group, consistent with previous data. As for IL-6 -174G/C polymorphism analysis, the significant positive correlation observed between G allele frequency and the patients' IL-6 levels and MAS prevalence may be evidence that the G allele is important for IL-6 synthesis. The higher IL-6 levels and MAS prevalence in

individuals with the GG genotype compared to those with the GC genotype were also key findings supporting this. It was not possible to analyze whether the IL-6 -597G/A polymorphism could be a risk factor for MAS development, because the AG genotype was detected in all patients in both the MAS and non-MAS groups. However, the fact that a considerable proportion of the limited number of studies investigating the -597G/A SNP failed to demonstrate a relationship with systemic diseases, taken together with the results of the present study, indicates that the -597G/A polymorphism does not have an important role in IL-6 synthesis.

The main limitation of this study was that the data were obtained from COVID-19 patients from a single race. As COVID-19 is a global problem, more useful results may be obtained with multi-population analyses evaluating the relationship with MAS, which is an important factor in COVID-related morbidity and mortality.

In conclusion, COVID-19 will likely continue to be a major issue, both due to the direct effects of the disease and its postinfectious sequelae. MAS is one of the leading risk factors for the development of these problems in COVID-19 patients. Identifying individuals with the -174G/C polymorphism of IL-6, which is an important cytokine in the development of MAS, and ensuring these individuals receive early immunization may help prevent COVID-related problems.

Compliance with Ethical Standards:

Conflict of interest statement: The authors received no financial support for the research and/or authorship of this article. The authors declare that they have no conflict of interest to the publication of this article.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee

and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This research was supported by the University Scientific Research Project Office (project number 6607).

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Figure legend

Figure 1. IL-6 (-174G/C) genotype significantly associated with serum IL-6 levels in COVID-19 patients. Serum IL-6 levels in COVID-19 patients (GC genotype: n=49, GG genotype: n=21) were measured by enzyme-linked immunosorbent assay. *, p=0.001 by independent-samples t test.

Table 1. Comparison of laboratory parameters between COVID-19 patients with and without MAS

	MAS patients (n=40) Mean ± SD	Non-MAS patients (n=30) Mean ± SD	p value
WBC (/µL)	8021.4 ± 6140.1	6410.2 ± 2214.5	**0.05**
Lymphocytes (/µL)	709.2 ± 316.2	1816.8 ± 881.4	**0.001**
Neutrophils (/µL)	6694.1 ± 6212.8	4417.3 ± 1889.9	**0.03**
NLR	12.2 ± 14.1	3.2 ± 2.7	**0.001**
AST (U/L)	43.3 ± 18.6	30.4 ± 22.4	**0.03**
ALT (U/L)	32.1 ± 29.4	30.1 ± 25.5	0.22
LDH (U/L)	512.1 ± 400.1	281.6 ± 138.5	**0.001**
GGT (U/L)	58.2 ± 38.8	36.1 ± 23.7	**0.02**
ALP (U/L)	82.1 ± 34.8	74.1 ± 41.4	0.43
Sodium (mmol/L)	137.1 ± 7.1	139.2 ± 3.2	0.4
Potassium (mmol/L)	4.1 ± 0.8	4.2 ± 0.4	0.8
Creatine (mg/dL)	1.9 ± 1.7	0.8 ± 0.6	**0.04**
Prothrombin time (s)	21.3 ± 12.5	13.4 ± 4.1	**0.02**
CRP (mg/dL)	182.1 ± 82.2	25.2 ± 22.4	**0.001**
Troponin-I (ng/dL)	282.2 ± 718.3	9.1 ± 20.4	**0.001**
PaO_2/FiO_2	219.8 ± 77.8	317.6 ± 50.8	**0.001**
D-dimer (ng/mL)	2623.9 ± 2117.7	666.2 ± 755.8	**0.03**
Ferritin (ng/mL)	1180.4 ± 1499.9	356.7 ± 164.1	**0.001**
IL-6 (pg/ml)	127.6 ± 95.6	32.2 ± 35.3	**0.001**

MAS: Macrophage activation syndrome, WBC: White blood cells, NLR: Neutrophil/lymphocyte ratio, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, LDH: Lactate dehydrogenase, GGT: Gamma glutamyl transferase, ALP: Alkaline phosphatase, SD: Standart deviation

Table 2. Interleukin-6 -174G/C allele/genotype frequencies and test of Hardy-Weinberg equilibrium in the MAS and non-MAS groups

	Non-MAS (n=30)		MAS (n=40)	
f(G)	0.55		0.73	
f(C)	0.45		0.27	
	O	E	O	E
CG	27	14.8	22	9.9
GG	3	9.1	18	21.3
	χ^2= 10.029, df= 1, p=0.002		χ^2= 9.998, df= 1, p=0.002	

Note: MAS: Macrophage activation syndrome, G: Guanine, C: Cytosine, f: Observed frequency of each allele (G or C), O: observed genotype numbers, E: Expected genotype numbers in the Hardy–Weinberg (HW) model, χ^2: Chi-square values, p: probability of difference

Table 3. Comparison of interleukin-6 (-174G/C, -597G/A) genotype frequency between the MAS and non-MAS groups

Interleukin-6 (-174G/C)				
	GC n (%)	GG n (%)	CC n (%)	p value
Non-MAS (n=30)	27 (90)	3 (10)	-	0.002
MAS (n=40)	22 (55)	18 (45)	-	
Interleukin-6 (-597G/A)				
	AG n (%)	GG n (%)	AA n (%)	p value
Non-MAS (n=30)	30 (100)	-	-	N/A
MAS (n=40)	40 (100)	-	-	

χ^2= 10, df= 1, MAS: Macrophage activation syndrome, A: Adenine, G: Guanine, C: Cytosine

Table 4. Comparison of interleukin-6 (-174G/C) allele frequencies between the MAS and non-MAS groups

	MAS (n=40)	Non-MAS (n=30)	p value
-174C allele (n)	22	27	0.032
-174G allele (n)	58	33	

χ^2= 4.615, df= 1, MAS: Macrophage activation syndrome, G: Guanine, C: Cytosine

Figure 1. IL-6 (-174G/C) genotype significantly associated with serum IL-6 levels in COVID-19 patients. Serum IL-6 levels in COVID-19 patients (GC genotype: n=49, GG genotype: n=21) were measured by enzyme-linked immunosorbent assay. *, p=0.001 by independent-samples t test.