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Frequency of Interleukin-6 rs 1800796 (-572G/C) and 2069837 (intron 2A/G), TNF-α rs1800750 (-376G/A), and 1800629 (-308G/A) polymorphism in COVID-19 patients with clinical degrees in Central Java

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ABSTRACT

Background: Coronavirus Disease 2019 (COVID-19) is an easily contagious disease, and not much is known about the characteristics of COVID-19, both in terms of susceptibility, severity, and spreadability of various SARS-CoV-2 strains. Patient genomic factors, especially related to genomic polymorphisms that affect the body's immune system, can influence the course of infectious diseases. The aim of this study is to get an adequate picture regarding gene polymorphisms, both susceptibility and related to the clinical degree in COVID-19 patients.
Methods: The PCR preparations were carried out in the Biomedical laboratory of the Faculty of Medicine, Universitas Sebelas Maret. The qualitative PCR (qPCR) examination was sent to the Genetica Science laboratory, Tangerang, West Java. The research subjects were divided into 3 groups, namely COVID-19 patients with no symptoms, COVID-19 patients with mild-moderate symptoms, COVID-19 patients with severe-critical symptoms. The research subjects were taken 6 cc of venous blood (3 cc for examination of serum IL-6 and TNFα levels and 3 cc for DNA examination).
Results: Serum levels of IL-6 and TNF-α in the clinical grade group were almost all above normal values. The frequency of TNF- α polymorphisms (-376G/A) all showed homozygote GG. TNF-α (-308G/A) also showed homozygote GG was dominant for SARS CoV2. IL-6 (-572G/C) polymorphism for cases requiring medium and severe clinical degree hospitalization was found to have more C allele than G allele. IL-6 polymorphism (intron A/G) the G allele is less common in cases requiring hospitalization.
Conclusion: TNF-α(−308A) allele has an influence on the development of clinical symptoms of SARS CoV2 infection. The rs1800796GG genotype in the IL-6 promoter contributes to milder symptoms in SARS CoV2 infection. Allelic variants of the gene under study may show different effects in other races depending on their interactions with other risk factors.

Keywords: COVID-19, clinical degree, IL-6, polymorphism, TNF-α.
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INTRODUCTION

The COVID-19 pandemic, which has emerged around the world, is spreading rapidly and causing death. COVID-19 patients may develop pneumonia, with severe symptoms of ARDS and multi-organ failure.[1] The SARS-CoV-2 protein attaches to the ACE2 receptor and then undergoes cleavage of the C-terminal ACE2 segment by transmembrane protease serine 2 (TMPRSS2) which will increase the viral spike protein into host cells. The genetic variability of ACE2 and TMPRSS2 determines the severity of COVID-19.[2] ARDS is the cause of death of COVID-19 patients, it is known that there is an increase in the number of proinflammatory cytokines including IL-6, IL-1β, TNF-α, IFN-γ and chemokines.[3] Genetic predisposition background causes individual disease susceptibility and severity.[2]

The number of cases that continues to grow is related to the difficulty of reducing the spread of COVID-19, so it is very important to carry out this research. This study aims to map genetics related to gene polymorphisms that play a role in the pathogenesis and severity of disease, and to create primers that are used to help identify the presence of gene polymorphisms both susceptibility and severity of COVID-19 infection in patients at the time of initial diagnosis so that they can be used as a guide for appropriate therapy and reduce mortality.

The specific purpose of this study was to determine the patient's genomic polymorphism and to determine its relationship to the susceptibility and clinical profile of COVID-19 patients. This research has a very strategic value, showing

a real contribution to the development of science and development in local, national, and international contexts, because the polymorphism data obtained is not only used for research related to COVID-19, but will also add data related to gene polymorphisms in Indonesia. Therefore, starting from Surakarta, we have the desire to research gene polymorphisms in the hope that it will help the genetic/molecular database of COVID-19 patients, and from that gene database we can make prototypes in the form of primers that are useful in treatment according to gene polymorphisms of COVID-19 patients.

METHODS

The study was conducted in the COVID-19 ward of the Universitas Sebelas Maret Hospital (UNS Hospital), Sukoharjo and the Haji Donohudan Dormitory in Boyolali, Central Java, from May to November 2021. The PCR preparations were carried out in the Biomedical laboratory of the Faculty of Medicine, Universitas Sebelas Maret. The qualitative PCR (qPCR) examination was sent to the Genetica Science laboratory, Tangerang, West Java. The research period is 12 months covering: preparation of tools and materials, sampling, sample processing, data analysis, and writing of results.

The information collected in this study included demographic, clinical history, laboratory test data, pharmacological treatment, and follow-up. The research subjects were confirmed COVID-19 patients in the COVID-19 ward of the UNS Hospital and the Haji Donohudan Boyolali Dormitory who were willing to be involved and signed the informed consent. Research subjects who meet the inclusion and exclusion criteria, their identity, other illnesses, etc. are recorded on the form provided. The research subjects were taken 6 cc of venous blood (3 cc for examination of serum IL-6 and TNFα levels and 3 cc for DNA examination). For asymptomatic cases, the study subjects were taken from the Haji Donohudan Boyolali Dormitory, patients who had confirmed COVID-19 were not subjected to a chest X-ray examination. The research sample was treated at UNS Hospital, a chest X-ray was performed, and an AGD examination was carried out specifically for severe and critical cases. This study followed the progress of the patient, i.e., recovered or died, if in the course of the disease the condition worsened, the clinical severity would be re-evaluated and 3 cc of serum would be taken again to be examined for IL-6 and TNFα serum. Then the blood was stored in a refrigerator at -80°C before DNA and serum tests were carried out. The research subjects were divided into 3 groups, namely COVID-19 patients with no symptoms, COVID-19 patients with mild-moderate symptoms, COVID-19 patients with severe-critical symptoms. The group is followed until the result is recovered or died.

Examination of serum interleukin-6 levels

The materials for the ELISA examination are: anti-human Il-6 96-well Strips Plate, recombinant Human IL-6 standards, standard diluent, Biotinylated antibody reagent, wash buffer. Streptavidin-HRP concentrate, streptavidin-HRP dilution buffer, TMB substrate, stop solution, and adhesive plate covers. The tools for ELISA examination are Elisa Reader, namely: micropippete, yellow and blue tips, rotator, centrifuge. Kit for ELISA with thermo scientific brand.

Serum TNFα level examination

The materials for the ELISA examination are: anti-human Il-1β 96-well Strips Plate, recombinant Human TNFα standards, standard diluent, Biotinylated antibody reagent, wash buffer. Streptavidin-HRP concentrate, streptavidin-HRP dilution buffer, TMB substrate, stop solution, and adhesive plate covers. The tools for ELISA examination are Elisa Reader, namely: micropippete, yellow and blue tips, rotator, centrifuge. kit for ELISA with thermo scientific brand. Examination of IL-6 and TNFα polymorphisms by quantitative PCR method.

Genotyping of allelic variants (single nucleotide polymorphism, SNPs)

The DNA samples were genotyped for polymorphisms IL-6 SNP gene rs1800795, rs2066992, rs2069869, IL-1β SNP gene rs3136558, rs16944, rs1143627 using kiCqStart SYBR® Green ReadyMixTM (Sigma Aldrich, Germany). The primers listed in Table 1. In brief, the procedure for the real-time PCR was the following: 15 ng DNA, 15 µL of Taqman universal PCR master mix (Roche NJ, USA) and 6.5 µL of each probes. The conditions for amplification were the following: 94°C (3 min), 61°C (1 min), and 72°C (1 min); followed by 35 cycles of 94°C (1 min), 61°C (1 min), and 72°C (1 min); and a final cycle of 94°C (1 min), 61°C (1 min), and 72°C (5 min).

Analysis

When the polymorphisms were evaluated, the ancestral genotype was used for comparison. The differences among the clinical parameters, continuous variables, and polymorphisms were evaluated by using the Chi-Square test. Software packages SPSS 19 (IBM, Chicago, IL) and Epi-Info 6.04b were used (Atlanta, CDC).

RESULTS

The study was conducted between May and November 2021, taken from UNS Hospital and Donohudan Hajj Dormitory. So far, 49 patients with a diagnosis of COVID-19 have been collected, which were determined based on the results of the PCR examination which showed positive SARS CoV-2 (Table 1-6). The DNA samples and genetic data of the single nucleotide polymorphisms (SNPs) are provided in Table 1 and Table 2. Patients who came from UNS Hospital were examined in the hospital emergency room, especially for chest X-ray examination and blood gas analysis, to determine the degree of disease. Meanwhile, patients from Haji Donohudan Dormitory were asymptomatic patients or mild symptoms.

The data obtained at this time from the patients who had been examined consisted of 49. Most had mild symptoms 17 (34.7%) and asymptomatic 13 (26.5%) followed by moderate symptoms 11 (22.4%), and severe symptoms 8 (16.3%) (Table 3-6). On the other hand, moderate cases must be treated in a hospital because the patient has pneumonia or pneumonia, so if there is respiratory failure. At this moderate level, the chance of recovery is still high, so it needs careful management. Patients with severe and critical degrees need to be treated in the intensive room, because respiratory and organ failure has occurred.

Table 1. DNA samples were genotyped by using Taqman commercial probes.

Gene (reference SNP)	Probe
TNF α (rs1800750)	GAGGCAATAAGACCCCCCTCGGAATC[A/G]GAGCAGCTGTCAATTGCAGGAGCT
TNF α (rs1800629)	GAGGCAATAGGTTTTGAGGGGCATG[A/G]GGACGGGGTTCAGCCTCCAGGGTCC
IL 6 (rs1800796)	AGTTCTACAACAGCC(C/G)CTCACAGGGAGAGCC
IL 6 (rs2069837)	GTGCCAGGCACTTTA(G/A)ATAAATATTGTGTCT

Table 2. Genetic data of the single nucleotide polymorphisms (SNPs) analyzed.

SNP	Gene			
	Symbol	Location	Position	Alleles
TNF (rs1800750)	TNF α	-376	Promoter	G/A
TNF (rs1800629)	TNF α	-308	Promoter	G/A
IL 6 (rs 1800796)	IL-6	-572	Promoter	G/C
IL 6 (rs2069837)	IL-6	Intron 2	Intron 2	A/G

Table 3. Distribution of TNF and IL-6 polymorphisms on clinical degrees of patients infected with SARS-CoV-2.

	Clinical Degree (n=49)			
	No Symptom n(%)	Mild n(%)	Medium n(%)	Severe n(%)
TNF α (-376G/A)				
GG	13(26.5)	17(34.7)	11(22.4)	8(16.3)
TNF α (-308G/A)				
GG	13(26.5)	17(34.7)	10(20.4)	8(16.3)
GA	0(0.0)	0(0.0)	1(2.0)	(0.0)
IL 6 (-572G/C)				
GC	2(4.1)	7(14.3)	2(4.1)	5(10.2)
CC	9(18.4)	5(10.2)	6(12.2)	3(6.1)
GG	2(4.1)	5(10.2)	3(6.1)	0(0.0)
IL 6 (intron2A/G)				
AG	4(8.2)	8(16.3)	1(2.0)	4(8.2)
AA	9(18.4)	7(14.3)	7(14.3)	4(8.2)
GG	0(0.0)	2(4.1)	3(6.1)	0(0.0)

Table 4. Mean levels of TNF and IL-6 by clinical degree.

	Clinical Degree			
	No Symptom Mean±SD	Mild Mean±SD	Medium Mean±SD	Severe Mean±SD
IL-6 (pg/mL)	9.97±4.67	16.01±11.04	45.52±46.30	22.49±23.16
TNF-α (pg/mL)	6.07±4.36	14.30±15.32	18.88±17.83	13.45±14.85

Table 5. Mean levels of TNF-α and IL-6 based on cure rate.

	Cure rate	
	Death Mean±SD	Cure Mean±SD
IL-6 (pg/mL)	13.84±8.80	22.32±27.80
TNF-α (pg/mL)	8.31±3.71	13.16±14.54

Based on Table 4, we can see that IL-6 is more expressed in a patient with no symptoms, mild, moderate, or even severe clinical findings than TNF-α. Mean levels of IL-6 are also more expressed in the patient based on the cure rate (Table 5).

In severe and critical degrees, treatment is needed in the ICU because there has been a respiratory failure or other organ failure or multi-organ failure. It is necessary to prepare for the use of a ventilator or other means of administering oxygen. Some drugs should be given such as anti-viral anti-inflammatory anti-coagulopathic antibiotics. Based on the data, the TNF α (-376G/A) and TNF α (308G/A) polymorphisms are more expressed in the cured patient (Table 6).

DISCUSSION

Cytokines are key protein regulators of the host response to infection and inflammation. They are small proteins having molecular weights ranging from 8 to 40 kDa. In humans, there is growing evidence that host cytokine responses are genetically determined. In healthy individuals, there are stable and reproducible differences in cytokine production, and these differences are related to genetic variations in encoding genes. Most cytokine genes are polymorphic, single nucleotide polymorphisms (SNPs). SNPs are substitutions of a single base at a specific site of a gene.[4] TNF-α and IL-6 have potent pro-inflammatory actions, exerting a pleiotropic effect on various cell types.[4,5]

Polymorphism studies for SARS CoV2 in Indonesia is still rare. This evaluation is aimed at cytokines that play an important role in the pathogenesis of SARS-CoV2 pneumonia, namely IL-6 (-572 G/C, intron 2A/G), and TNF-α (-376G/A, -308G/A). Interleukin 6(-572 G/C) has previously been known to be associated with respiratory tract infections, such as tuberculosis, as well as with viral infections of hepatocytes.[6,7] Meanwhile, IL-6 (second intron of IL-6) is known to be associated with SARS CoV2 infection.[8] TNF-α(-376G/A) is also known to be associated with SARS CoV2, while TNF- α(-308G/A) is associated with upper respiratory tract infections.[4,9,10]

The evaluation was more focused on knowing the relationship of polymorphisms to the clinical degree

Table 6. TNF and IL-6 polymorphisms based on cure rate.

	Cure Rate (n=49)	
	Death n(%)	Cure n(%)
TNF α (-376G/A)		
GG	3 (6.1)	46 (93.9)
TNF α (308G/A)		
GG	3 (6.1)	45(91.9)
GA	0(0.0)	1(2.0)
IL 6 (-572G/C)		
GC	1(2.0)	15(30.6)
CC	2(4.1)	21(42.9)
GG	0(0.0)	10(20.4)
IL 6 (intron2A/G)		
AG	1(2.0)	16(32.7)
AA	2(4.1)	25(51.0)
GG	0(0.0)	5(10.2)

of SARS CoV2 infection by classifying patients no symptoms and mild symptoms who could be treated as outpatients with moderate and severe patients requiring hospitalization. In this study, 49 patients underwent polymorphism examination, with 30 (61.2%) being outpatient and 19 (38.72%) requiring hospitalization.

ELISA serum examination results showed that the mean levels of IL-6 and TNF-α in the clinical grade group were almost all above normal values, except for TNF-α levels in the asymptomatic group. TNF-α and IL-6 normal upper limit values were 8.1 pg/mL and 7 pg/ml, respectively.[6,11]

Patients requiring hospitalization for moderate and severe clinical degree showed higher mean values of both IL-6 and TNF-α than no symptom and mild clinical degree patients. High levels of IL-6 are found in patients with more severe clinical degrees.[12,13] High levels of IL-6 can interfere with tissue perfusion and cause endothelial damage, as well as the formation of micro thrombus which increases vascular permeability, increases vascular permeability, this causes fluid to accumulate in lung tissue in the interstitial spaces clinically manifests as acute respiratory failure.

However, we found that the frequency of TNF-α polymorphisms (-376G/A) all showed homozygote GG. Meanwhile, TNF-α (-308G/A) also showed homozygote GG was dominant for SARS CoV2 to occur, both in no symptom to a severe clinical degree patients. Similar results were obtained from Sotomayor's *et al.* report that TNF-α (-308G/A) showed that people carrying the A allele not more susceptible to infection with SARS-CoV-2.

The low frequency of the A allele and TNF-α (-376G/A) AA Polymorphism in this population may be due to natural selection preferring individuals with low production potential to individuals with high TNFα secretion, so that the A allele is rare in cases of SARS CoV2 infection.[5]

Different results were reported by Saleh A et al., 2020 that people carrying the A allele (AA and AG) are more susceptible to SARS CoV2 infection, even in the study it was stated that the AA genotype was associated with a more aggressive disease and a less favorable disease course compared to other genotypes.[4] The reason for this difference is probably because we did not control for allele and genotype frequencies according to ethnic background.[5]

The IL-6 (-572G/C) polymorphism for cases requiring medium and severe clinical degree hospitalization was found to have more C allele than G allele. The same thing also happened to IL-6 (intron A/G) for cases requiring hospitalization, the allele A is more abundant than the G allele. This means that for the IL-6 polymorphism (intron A/G) the G allele is less common in cases requiring hospitalization.

This is different from the study, which showed that IL-6(597G/A) polymorphism and IL-6 levels showed no significant correlation with IL-6 (-597G/A) polymorphism.[12] However, for another polymorphism, IL-6(-174G/C) polymorphism, it showed that severe SARS CoV2 cases correlated with the G allele frequency. In addition, serum IL-6 levels were higher in individuals with GG genotype than in the GC genotype.[12] This difference occurred because the data were obtained from SARS-CoV-2 cases from one race. As the case of SARS CoV2 is a global problem, it would be more useful to carry out a multi-population analysis evaluating the correlation of IL-6 gene polymorphisms.

The G allele tends to a milder clinical degree. It is suspected that the G allele can decrease IL-6 expression by blocking the binding of MEF2a (Myocyte Enhancer Factor 2a) and increasing the anti-inflammatory gene GPNMB (Glycoprotein Nonmetastatic Melanoma Protein B). Changes from A to G will decrease luciferase activity which can decrease IL-6 transcription, so it can be said that the G allele will play a positive role against SARS CoV2 by reducing IL-6 transcription.[8] This is in accordance with this study, that the G allele was found in milder symptoms.

CONCLUSION

Genetic variance is a non-modifiable risk factor and cannot be claimed to be translated directly to the clinic as an actionable marker in the current emergency. TNF-α(−308A) allele has an influence on the development of clinical symptoms of SARS CoV2 infection. The rs1800796GG genotype in the IL-6 promoter contributes to milder symptoms in SARS CoV2 infection. Allelic variants of the gene under study may show different effects in other races depending on their interactions with other risk factors. Studies are replicated in different ethnicities and larger populations and are needed to validate our results.

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CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

AUTHOR CONTRIBUTION

All authors contributed equally in conducting the study as well as writing and revising the manuscript.

ETHICS CONSIDERATION

This study was approved by the Medical Research Ethics Committee No: 46/UN27.06.6.1/KEP/EC/2021, issued by the Faculty of Medicine UNS.

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