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POLYMORPHISMS IN THE ACE2 AND IL-6 GENES AND THEIR POTENTIAL IMPACT ON THE SUSCEPTIBILITY OF SEVERE COVID-19 AMONG ERBIL HOSPITALIZED PATIENTS.

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ABSTRACT:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a transmissible illness caused worldwide pandemic. This virus invades host cells via receptors of angiotensin-converting enzyme 2 (ACE2). Moreover, the viral infection stimulates the production of a variety of cytokines like interleukin-6 (IL-6). The main aim of this work is to investigate the connection between COVID-19 and polymorphism of *ACE2* (rs2106809 and rs2285666) and *IL-6* (-174 G/C) (rs1800795) genes in a group of patients. Genomic DNA was prepared from the peripheral blood of 60 hospitalized patients and 22 controls, the *ACE2* and *IL-6* genes were amplified by PCR, and the products were sequenced. The data demonstrated a significant variation in the genotype frequency of *ACE2* between COVID-19 patients and healthy subjects.The *ACE2* (rs2106809) polymorphism outcomes expressed the frequency of three genotypes (TT, TC, and CC), the patients with the TC allele are at risk of developing the disease by approximately 8-folds (OR= 7.5) compared to those with TT and CC alleles. Furthermore, no significant association was found between *ACE2* (rs2285666) polymorphism and the risk of developing SARS-CoV-2 which showed a frequency of (AA, AG, and GG) alleles. Additionally, there was no noticeable linkage between the (GG, CC, and CG) genotypes of *IL-6* (−174 G/C) (rs1800795) and the hazard of contracting COVID-19. In conclusion, this investigation confirmed that the TC genotype of *ACE2* (rs2106809) polymorphism represents a risk factor for acquiring COVID-19 and proposed to perform a critical action in the severity of pathogenicity in Iraqi Kurdish people.

KEYWORDS: COVID-19, ACE2; Angiotensin-converting enzyme 2, IL-6; Interleukin -6, Polymorphisms.

1. INTRODUCTION

The coronavirus sickness 2019 (COVID-19), which is caused by the SARS-CoV2 virus, was declared by WHO on January 12, 2020 (Liu *et al*., 2020). After analyzing a cluster of pneumonia cases from an unidentified source in 2019, researchers from China found a novel Coronavirus. As a result of over 80% genetic similarities with SARS-CoV, the novel Coronavirus was initially dubbed 2019-novel CoV (2019-nCoV). Later, the Coronavirus Study Group of the International Committee on Taxonomy of Viruses designates the virus as SARS-CoV-2 *(*Wang *et al*., 2020).

The SARS-CoV-2 was expected to arise by the end of 2019 via zoonotic transmission through a bat reservoir, leading to an epidemic (Zhou *et al*., 2020). This virus is attached to the angiotensin-converting enzyme 2 (*ACE2*) of the host cells. Since ACE2 is more found in the alveolar epithelial type II cells, it is possible to explain why the lungs are the main site of COVID-19 effects (Sun *et al*., 2020).

The gene coding for ACE2 *(ACE2)* in humans, is located on chromosome Xp22.2 and consists of 41,116 base pairs. (Turner and Hooper, 2002 & Lippi *et al.*, 2020). Angiotensin I (Ang I) and Ang II are cleaved by ACE2 into the inactive peptides Ang (1-9) and Ang (1-7), correspondingly. Ang (1–9) gets metabolized into Ang (1–7) which performs as a vasodilator. This means that ACE2 is able to counteract the vasoconstrictive impacts of the ACE-Ang II axis. (Zhang *et al*., 2020).

Acute respiratory distress syndrome (z) development and progression are closely correlated with the inflammatory cytokine storm that arises after SARS-CoV-2 infection. Affected people diagnosed with ARDS exhibit a substantial rise in cytokine levels in their sera. There is a direct relationship between the degree of the rise and the rate of mortality (Parsons *et al.*, 2005).

IL-6 is critically important for the initiation and progression of a cytokine storm, and in numerous recent clinical trials, the levels in the severe patients were higher than those in the moderate ones. (Chen *et al.*, 2020). The glycoprotein IL-6, which is 21 KDa in size, is produced by the IL-6 gene in humans. It can function by the way of an anti-inflammatory myokine besides a pro-inflammatory cytokine. The gene that encodes IL-6 is located on chromosome 7p21 (Tanaka *et al.*, 2014). The polymorphism in the *IL-6* (−174 G/C) (rs1800795) allele is linked with a higher amount of IL-6 creation and pneumonia intensity in coronavirus-19-afflicted individuals (Ulhaq & Soraya, 2020).

Recently, increasing evidence proposes that genetic dissimilarities in the genes of *ACE2* and *IL-6* may modify the intermolecular contacts with coronavirus spike protein and share in lung and systemic damage by promoting inflammation, vasoconstriction, fibrosis, and oxidation. Also, it is worthwhile to note that no previous studies have been conducted in the provinces of Kurdistan-Iraq on the genetic polymorphism of *ACE2* and *IL-6* genes among COVID-19 patients. Therefore, this study aims to investigate the polymorphism of ACE2 (rs2106809 and rs2285666) and IL-6 (-174 G/C) (rs1800795) genes in severely hospitalized COVID-19 patients of Erbil, Iraqi.

2.MATERIALS AND METHODS

2.1. Samples selection and collection

COVID-19 hospitalized patients (n= 60) of various ages and both sexes aged (20-70) years were enrolled in this study

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from both West Erbil Emergency Hospital and the COVID-19 control center in Soran/Erbil, Iraq. Twenty-two healthy subjects of similar age and sex have been randomly chosen from healthy Soran city residents to serve as the control group.

2.2 Ethics approval

This work was carried out with the patient's relatives' verbal and written approval before the sample was taken. A scientific ethics committee reviewed and approved the study proposal at the Faculty of Science, Soran University, and the official form 1/1/178 with the date of 15/11/2021.

2.3 Extraction of DNA

Two mls of blood were collected in EDTA tubes from both groups (Patients and Healthy) and the extraction of DNA was accomplished by the ReliaPrep™ Blood gDNA Miniprep System. This method was performed following the Promega Technical Manual procedure kit. Electrophoresis was applied to study the extracted DNA from the blood of both the control group and patients with COVID-19 on agarose gel. The gel used in this study had a composition of agarose (0.8%) besides ethidium bromide (0.5 μg/ml). The samples were viewed under UV light (300 nm) to visualize the results (Sambrook & Russell, 2001).

2.4 Analysis of genetic variations in *ACE2* and *IL-6* SNPs

To detect *ACE2* (rs2106809), (rs2285666), and *IL-6* (−174 G/C) (rs1800795) polymorphisms. PCR was carried out for every gene with a pair of forward and reverse primers (Karakaş Çelik *et al.*, 2021; Falahi *et al.*, 2022), as in Table 1.

Table 1: Primer sequences of *ACE2* (rs2106809), (rs2285666), and *IL-6* (-174 G/C) (rs1800795) polymorphisms.

Primers	Sequence	Product Size
ACE2 (rs-2106809)	F: 5`-GAAAGCCAGATGCTTTAACAAG-3`	207 bp
	R: 5`-TTTTTCCATATCTCATCTGATCG-3`	
ACE2 (rs-2285666)	F: 5`-CATGTGGTCAAAAGGATATC-3`	466 bp
	R: 5`-AAAGTAAGGTTGGCAGACAT-3`	
IL-6 (−174 G/C) (rs1800795)	F:5′-TTGTCAAGACATGCCAAGTGCT-3′	300 bp
	R: 5′- GCCTCAGAGACATCTCCAGTCC-3′	

The procedure outlined in Table 2 was followed for the PCR and the reaction was prepared depending on the manufacturer's directions for PCR PreMix (Bioneer, Korea) (Technologies Corporation, 2014). Twenty-five microliter of PCR product of each sample was sequenced. The automated DNA sequencer used was the ABI3730XL by (Macrogen Corporation – Korea) to sequence the sample using Sanger's technique. The sequences were handled and investigated by the Basic Local Alignment Search Tool (BLAST) to explore the homologous sequences in the database of the National Centre for Biotechnology Information (NCBI) .

Table 2: PCR Thermo cycling condition for *ACE2* (rs-2106809 and rs-2285666) and *IL-6* (-174 G/C) (rs1800795)

Steps	Temperature	Time	No. of cycles
Initial denaturation	94 ˚C	5 minutes	30 cycles
Denaturation	94 ˚C	30 second	
Annealing	55.5 ˚C *ACE2* (rs-2106809) 57.3 ˚C *ACE2* (rs-2285666) 55.3˚C *IL-6* (-174G/C) (rs1800795)	30 second	
Extension	72˚C	30 second	
Final extension	72 ˚C	5 minutes	
Hold	8 ˚C	∞	1 cycle

2.5 Statistical Analysis

Descriptive statistics have been performed to display the characteristics of patients by using percentages. The calculation and analysis of odds ratios (ORs) and the 95% confidence levels (CLs) were accomplished by the Statistical Package for the Social Sciences (SPSS).

3. RESULTS

As shown in Figure 1. A, the genomic DNA that was obtained and subjected to electrophoresis using a 0.8% agarose gel. Additionally, electrophoresis was performed on an agarose gel of 2 % to detect the products of the PCR, as shown in Figure 1. B, C, and D.

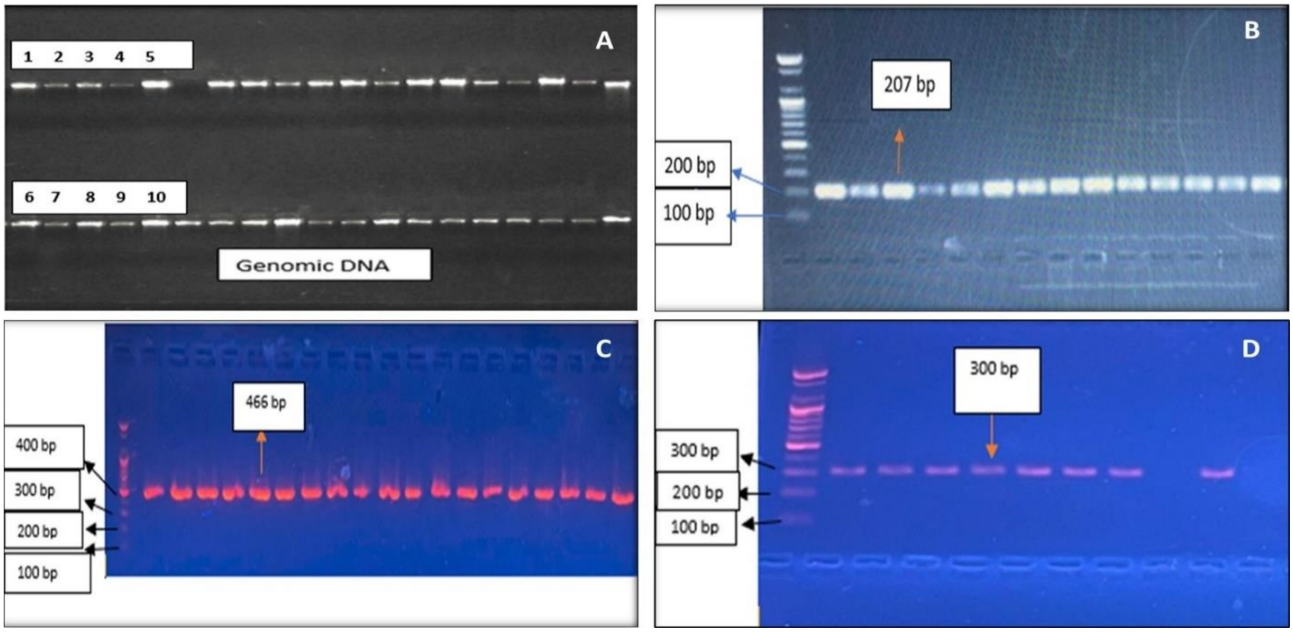

Figure 1: A. Genomic DNA extracted from COVID-19 patients (1-5) and healthy controls (6-10) in agarose gel. B. Agarose gel of PCR amplified 207 bp of *ACE2* (rs-2106809) SNP stained with ethidium bromide in patients and control samples. C. Agarose gel of PCR amplified 466 bp of *ACE2* (rs-2285666) stained by ethidium bromide in patients and control samples. D. Agarose gel and detecting PCR amplification of 300 bp of *IL-6* (-174 G/C) (rs1800795) in both patient and control samples stained by ethidium bromide. Compared to the DNA Ladder 100 bp

The *ACE2* (rs2106809) polymorphism results showed the frequency of homozygote (TT) was (36), while the frequency of heterozygote (TC) was (16); however, the homozygote (CC) represented the lowest frequency in SARS-CoV-2 patients. The results of the control group also showed a dominance of the (TT) genotype (n=17). The (TC) was found in one participant and the (CC) genotype was found in four samples. A considerable relationship between the *ACE2* (rs2106809) polymorphism genotype and the hazard of getting coronavirus-19 was observed in this investigation under $p< 0.05$. Patients who have the TC allele will be at risk of developing the disease about 8-fold (OR= 7.5) compared to the TT allele. Table 3 displays the p-value, 95% CI intervals, and odd ratio.

Table 3: *ACE2* (rs2106809) genotypes frequency detected in COVID-19 patients and healthy subjects.

Genotype	Control group (n=22)	Patients (n=60)	OR	95% CI	P- Value
TT	17	36	1.0	-	-
TC	1	16	7.5	1.01- 61.762	0.031
CC	4	8	0.94	0.249 - 3.577	1.00

The *ACE2* (rs2285666) polymorphism results showed the frequency of homozygote (GG) was (36), while the frequency of heterozygote (AG) was (21): nevertheless, the homozygote (AA) represented the lowest frequency in SARS-CoV-2 patients. Furthermore, the findings of (22) samples in the control group showed the dominance of the (GG) genotype (n=18). The (AA) was found in (4) participants, and no genotype was found (AG).

The current investigation demonstrated no significant correlation between the polymorphism in *ACE2* (rs2285666) and the hazard of acquiring SARS-CoV-2 under $p< 0.05$ probability. The p-value of the 95% CI intervals and the odd ratio, as shown in Table 4.

Table 4: The frequency of *ACE2* (rs2285666) genotypes detected in COVID-19 patients and healthy control.

Genotype	Control group (n=22)	Patients (n=60)	OR	95% CI	P- Value
AA	4	3	1.0	-	-
AG	0	21	0.0754	1.415- 423.98	0.027
GG	18	36	0.333	0.100- 11.42	0.072

The *IL-6* (174 G/C) (rs1800795) polymorphism results indicate, that the frequency of heterozygote (CG) was (42), while the frequency of homozygote (CC) was (14); however, the homozygote (GG) represented the lowest frequency in COVID-19 patients, appearing in only 4 cases. In the control group, the outcomes of (22) samples also recorded the (CC) genotype as (11). The (CG) was found in (10) participants, and one genotype was found in (GG). In this study, it was observed that the danger

of getting COVID-19 didn't have a noteworthy association with polymorphism of IL-6 (-174 G/C) (rs1800795) at a probability less than 0.05. Table 5 displays the p-value, the 95% CI intervals, and the odd ratio.

Table 5: *IL-6* (−174 G/C) (rs1800795) genotypes frequency detected in COVID-19 patients and healthy control.

Genotype	Control group (n=22)	Patients (n=60)	OR	95% Cl	P- Value
GG	1	4	1.0	-	-
CC	11	14	0.304	0.031 - 3.268	0.023
CG	10	42	1.1	0.106 - 10.444	0.044

DISCUSSION

In light of the achievements in the human genome development besides advancements in technology, scientists have been able to identify genetic variants more rapidly. There are various types of genetic variations, one of them is the single nucleotide polymorphism (SNP), which is more prevalent than mutations in the genetic material. The attachment and the invasion of the microbe to the host cell, vulnerability of the host, resistance to the infection, and the virulence of the pathogen are influenced by genetic polymorphisms. Many of these pathways have been revealed to be active (Öztürk *et al*., 2020).

According to the outcome of the current investigation, there is a statistically noticeable connection between the *ACE2* (rs2106809) polymorphism and the danger of coronavirus-19 with three genotypes (TT, TC, and CC). Moreover, it was observed that Patients who have the TC allele will be at risk of developing the disease about 8 folds (OR= 7.5) compared to the TT allele. These findings align with the results achieved by (Ciaglia *et al*., 2020) which revealed that individuals carrying the TC or CC genotype of *ACE2* (rs2106809) exhibited elevated amounts of ACE2 receptors in their circulation compared to individuals with the TT genotype.

Recent investigations have shown that *ACE2* single-nucleotide polymorphisms can influence SARS-CoV-2 susceptibility and clinical outcomes by affecting the binding affinity and the degree of expression (Devaux *et al.*, 2020 & Çelik *et al.*, 2021). Also, Chaudhary (2020) has suggested that genetic factors may regulate the variation in circulating ACE2 levels, with the (rs2106809) polymorphism potentially influencing ACE2 levels. When compared to the TT genotype, circulating ACE2 levels were higher in individuals with CC or CT genotypes.

Our results disagree with (Çelik *et al*., 2021), who observed no correlation between the *ACE2* (rs2106809) and (rs2285666) polymorphisms and the harshness of COVID-19 symptoms. This discrepancy may arise from the different sample sizes enrolled in these studies.

Herein we discovered no important joining between the polymorphism of *ACE2* (rs2285666) and the danger of developing COVID-19, the analysis also identified three genotypes: AA, which refers to the wild-type genotype, AG, which denotes the mutant heterozygote genotype, and GG, which refers to the mutant homozygote genotype. Similarly, Çelik *et al*. (2021) did not observe any correlation between the rs2285666 variant and the clinical course of Coronavirus-2 in a cohort of 155 patients. This result is consistent with the current research as well as with a study conducted by Jevnikar *et al.* (2022). The latter study didn't find any connection between the allele frequencies or genotype distribution of the selected SNPs (rs2285666) and the occurrence or severity of COVID-19. Also, a study conducted by Najafi and Mahdavi examined coronavirus-19 patients distributed among three grades: severe, moderate, and mild. They found no correlation between the ACE2 (rs2285666) SNP and disease severity (Najafi and Mahdavi, 2023).

In contrast, Srivastava *et al.* (2020) showed that a reduced infection incidence and case-fatality rate were associated with (rs2285666) in Indian populations. A single nucleotide polymorphism (rs2285666) is associated with increased ACE2 synthesis with a higher affinity for SARS-CoV-2 (Pouladi & Abdolahi, 2021).

According to our research, the GG genotype is the most common among COVID-19 patients in Iraq, which is reliable with earlier research (Allami *et al.*, 2023). They found that 65.67% of patients had the GG genotype, while the mutant homozygous (AA) genotype was identified in 11.9% of patients. This suggests a possible occurrence of *ACE2* (rs2285666) and COVID-19 in Iraqi patients. Likewise, an earlier meta-analysis study revealed that patients who exhibited the genotype of (rs2285666) AG have a protective effect against severe COVID-19, whereas those with the (rs2285666) GG genotype have an increased hazard of development of acute symptoms (Keikha & Karbalaei, 2022).

In an investigation conducted by Sidhwani et al., it was found that the most frequent variation observed was rs2285666. This variation was found in Pakistani COVID-19 patients. The research indicated that nearly half (49.2%) of the participants possessed the CC genotype, while 45.2% had the TT genotype. About 4.8% of individuals exhibited CT heterozygosity, and only 0.8% of them were carriers of the AA genotype (Sidhwani *et al.*, 2023). Nevertheless, it is important to note that the factors contributing to the variation among the above-mentioned research are likely to be ethnicity, the technique used in the research and sample size.

It has been found through several investigations, that gene polymorphisms of *IL-6* at rs1800795 (174 G / C) are linked to the levels of IL-6 in the blood as well as the prevalence, and/or progression of a wide range of diseases, *IL-6* polymorphisms can act as indications of the severity in patients with COVID-19 or individuals who are asymptomatic (Vitkauskaite *et al.*, 2021, Kirtipal & Bharadwaj, 2021), Newly discovered evidence indicates that higher inflammatory patterns in the outcome of SARS-CoV-2 pneumonia are linked to *IL-6* (174). The direct effect of *IL-6* (174) on the fate of CD8 and CD4 T cells may explain this occurrence (Rokni *et al.*, 2022).

The present research observed no significant connection between the polymorphism of *IL-6* (rs1800795) and the SARS-CoV-2 danger. It also revealed the presence of three genotypes: GG for the wild-type genotype, CG for the mutant heterozygote genotype, and CC for the mutant homozygote genotype. This work is consistent with the study of Abed *et al.*, (2023), who determined that the variations in a gene coding for *IL-6* at -174 G/C location might not serve as hazard aetiology for COVID-19 in patients from Iraq.

Falahi *et al.*, (2022) performed a research that found no notable variances in the genotype and allele occurrences of *IL-6* (rs1800795) SNP between two groups of COVID-19 patients from the Kurdish people of Iran. discovered that there were no noteworthy variations in both genotype and allele occurrences of *IL-6* (rs1800795) SNP between two groups of COVID-19 patients belonging to the Kurdish population of Iran. According to another study, *IL-6* rs1800795 genotyping of (G/C) exhibited expression of all genotypes in entire groups. Nevertheless, IL-6 rs1800795 CC genotype occurred additional frequently in mild COVID-19 patients (52.5%) and negative controls (60%) than in severe COVID-19 individuals (26.3%) (Ghazy, 2023).

In contrast to the current study's findings, the *IL-6* gene's polymorphisms at location (-174 G/C) have been linked to the

risk of SARS-CoV-2. This indicates that in Iraqi Arab people, this gene can be a major risk reason for the new coronavirus (Dhabaan *et al.*, 2022).

Additionally, in a recent study conducted on the Turkish population, a notable connection was discovered between the *IL-6* polymorphism in the location of 174 G/C (rs1800795) and SARS-CoV-2, which contradicts our present study (Fischchuk *et al.*, 2021). Furthermore, research conducted by (Kerget & Kerget, 2021) suggested that the presence of both genotypes G allele and GG of *IL-6* is highly combined with a raised vulnerability to COVID-19. This genetic factor indicates a higher risk of contracting the virus.

The investigation reveals that the CG genotype of *IL-6* (rs1800795) polymorphism was the most common among COVID-19 patients, which aligns with the findings of Dhabaan *et al.*, (2022) who demonstrated that *IL-6*-174 G/C polymorphisms indicate the G, as well as GG genotype, consider causal factors, while the C allele and CG genotype are protective ones that are combined with the hazard of coronavirus-19 in the Iraqi people. The variants of the *IL-6* gene, specifically rs1800795G, and the *IL-8* gene, specifically rs2227306C, have a robust association with severe outcomes of COVID-19. This association is particularly observed when both variants are present together. These variants can be potentially utilized as prognostic markers for COVID-19 (Ghazy,2023).

CONCLUSIONS

This stusy is one of the few that examines how differences in the *IL-6* and *ACE2* genes might be used to predict the severity of coronavirus-19 among patients of Kurdistan-Iraq. Although there were limitations in the investigation, such as sample number, ethnicity, and genetic polymorphism choice, we established a connection between the *ACE2* (rs2106809) SNP and the possibility of experiencing acute COVID-19 symptoms in the Kurdish community.

Also, there was no correlation between *ACE2* (rs2285666) and *IL-6* (174 G/C) (rs1800795) gene polymorphisms and SARS-CoV-2 disease severity. Additional genetic investigations incorporating a greater number of (SNPs) and a larger cohort are necessary to elucidate and validate the joining of SARS-COV-2 with genetic variants.

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