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Association of Apolipoprotein e polymorphism with SARS-CoV-2 infection

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ABSTRACT

Coronavirus 2019 (COVID-19) is a viral disease caused by severe acute respiratory syndrome coronavirus-2 (SARS CoV-2). The disease resulted in global morbidity and mortality that led to considering as pandemic. The human body response to COVID-19 infection was massively different from being asymptomatic to developing severe symptoms. Host genetic factors are thought to be one of the reasons for these disparities in body responses. Few studies have suggested that Apolipoprotein Epsilon (Apo E) is a candidate gene for playing roles in the development of the disease symptoms. This work aims to find an association between different Apo E genotypes and alleles to COVID-19 infection comparing a general population and a group of COVID-19 patients. For the first time, the results found that Apo E4 is associated with COVID-19 disease in a Kurdish population of Iraq. Further study is required to reveal this association in different ethnic backgrounds all over the world.

1. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection was declared a pandemic by WHO in March 2020 (WHO, 2020). It has been observed that SARS-CoV-2 infected individuals show massive differences in their response to the infection. The human body response was ranging from being asymptomatic in a large proportion of infected individuals (Yanes-Lane et al., 2020), to being mild, or to develop severe symptoms (Wu and McGoogan, 2020). The reasons behind these disparities in the severity of coronavirus disease 2019 (COVID-19) infection are yet unknown. Early at the beginning of the pandemic, it has been revealed that demographic factors like older age and male gender are risk factors for acquiring the infection and developing more severe symptoms (Jin et al., 2020). Then different pre-existing comorbidities are suggested as risk factors; these were including cardiovascular diseases (CVD), chronic respiratory disease (CRD), obesity, Cancer (Bajgain et al., 2021; Gasmi et al., 2021; Zheng et al., 2020), Alzheimer’s, dementia (Wang et al., 2021a), type 2 diabetes (DM), chronic obstructive pulmonary disease (COPD), pneumonia, atrial fibrillation (AF), chronic kidney disease (CKD) and hypertension (HTN) (Atkins et al., 2020).

Despite the demographic factors and pre-existing comorbidities the basic question regarding the differential susceptibility to COVID-19 infection and the development of symptoms has remained unanswered. However, one possible answer could be the host genetic background, therefore; the host genetic risk factors for acquiring infection and developing symptoms should be considered. Recently, a large number of studies have been directed toward finding genes that confer differences in susceptibility for COVID-19 infection (Schurr, 2020). Some of the genetic association studies are performed based on the comparison of biobank genetic data (Kuo et al., 2020a). Most of these studies have revealed genetic risk factors, however, each study found different genes from other studies (Hu et al., 2020). Several genes have been suggested for possible association to COVID-19 acquisition and severity; these was including; Angiotensin converting enzyme 2 (ACE2), trans-membrane protease serine 2 (TMPRSS2) (Hou et al., 2020), interferon-induced transmembrane protein 3 (IFITM3) (1C and BH, 2020), ABO blood type (Cordero et al., 2021), and Toll-like receptor 7 (Fallerini et al., 2021).

One of the genetic risk factors, found to be associated with COVID-19 infection in a community cohort study, was Apolipoprotein E (Apo E) (Kuo et al., 2020a). Apo E is a lipoprotein that is related to the metabolism of lipids in the human body and it is also associated with both cardiovascular and neurodegenerative diseases including Alzheimer’s disease (Poirier et al., 1993). Apo E gene is polymorphic having three different alleles, E2, E3 and E4, with different frequencies (5–10%, 65–70% and 15–20%, respectively) among the general population (Mahley, 2016). The combination of these alleles gives rise to three

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homogenous and three heterozygous genotypes including E2/E2, E3/E3, E4/E4, E2/E3, E2/E4, and E3/E4 (Mahley, 2016). Apo E is found to be related to susceptibility to parasitic, bacterial and viral infections. Apo E4 allele has been associated with the greater risk of acquisition and severity of viral infections, like hepatitis C, Herpes simplex, and HIV (Burt et al., 2008; Kuhlmann et al., 2010). It was shown that the Apo E deficiency caused immune response impairment to certain bacterial infections (De Bont et al., 2000; Roselaar and Daugherty, 1998). There are also a growing number of studies showing that the Apo E gene is associated with pulmonary inflammation and oxidative stress. For instance, the E4 allele is found to be related to increasing pro-inflammatory cytokines, while Apo E3 has immune-modulatory roles in inflammation, (Figueroa et al., 2018) which is a major problem in COVID-19 patients.

Interestingly, Apo E polymorphism is also a genetic risk factor in most of the comorbidities related to SARS-COV-2 severity. Apo E polymorphism is found associated with cardiovascular diseases, type-2 Diabetes (Liu et al., 2019), Alzheimer’s disease, and Dementia (Wang et al., 2021a).

Before the COVID-19 pandemic happened, we had been studying genetic polymorphisms of Apo E alleles in a general population in Sulaymaniyah province, Kurdistan Region of Iraq (Al-Jaf, 2021). In an attempt to find a possible guilty gene for COVID-19 infection, we have designed a cohort study to seek an association between Apo E polymorphism and COVID-19 infection.

2. Materials and methods

2.1. Study subjects

Previously we have investigated the frequency of Apo E alleles in one hundred fourteen healthy individuals in the same area (Al-Jaf, 2021); the data from this study was used to represent the control group for the current study. From July to December/2020, One-hundred and five patients who visited COVID-19 clinics in Kalar district (Sulaimaniyah province, Iraq) were selected to represent COVID-19 cases. All the cases were confirmed by RT-PCR from nasopharyngeal swabs. Signed consent forms were filled for each patient. Venous blood samples were taken from each subject and kept in a refrigerator at 4 °C until used.

A detailed questionnaire was designed to collect all the necessary data from the patient’s records such as age, sex, and the clinical history for comorbidities like; asthma, diabetes, hypertension, ischemic heart disease and stroke. Acute-phase parameters including CRP, ESR, D-dimer and serum ferritin were also collected. PCR positive test, CT scan and SPO2 were also documented in the questionnaire.

2.2. DNA extraction

Total genomic DNA was extracted from whole venous blood by mixing 200 μl of each blood sample with 20 μl protease K and incubated at 56 ºC for 10 min, then the manufacturer’s (GENET BIO Co., Daejeon, KR) instructions were followed. The genomic DNA was eluted with a 200 μl elution buffer. The extracted genomic DNA samples were kept at –30 ºC until used.

2.3. Apo E genotyping

In this study, a sequence-specific primer method was used for Apo E genotyping. This method is a PCR based method in which four allele-specific primers (Macrogen Co., Seoul, KR) were used for the identification of Apo E isoforms (Forward Primer-1; CGG ACA TGG AGG ACG TGT for Apo E-112cys, Reverse Primer-2; CTG GTA CAC TGC CAG GCG for Apo E-158arg, Reverse Primer-3 CTG GTA CAC TGC CAG GCA for Apo E-158cys, Forward Primer-4 CGG ACA TGG AGG ACG TGC for Apo E-112arg, these primers were developed and validated previously (Pantelidis et al., 2003), and recently used in our lab for Apo E genotyping in a general population (Al-Jaf, 2021). Combinations of two allele-specific sequence-specific primers were used to determine each Apo E haplotype: primers F1 and R3 for E2, primers F1 and R2 for E3 and primers R2 and F4 for E4 haplotypes. Three PCR reactions were required to confirm the genotyping of each DNA sample. In each PCR reaction, in addition to Apo E primers, a pair of other primers were added to amplify a 406 bp region on chromosome 9 (Niranjani, 2020), this was used as a positive control to ensure that the PCR reactions are working. A thermal cycler (Nexuss, Eppendorf AG, Germany) was used to perform PCR reactions. A volume of 11 μl of a reaction mixture was prepared as follow: 5 μl of mastermix (Addbio, South Korea), 5 μl of sample DNA, and 0.8 μl (10 pmol) of allele-specific primers (0.4 μl of each forward and reverse primer) and 0.1 (10 pmol) μl of each control primers. The negative control was performed for each experiment by adding 5 μl of water instead of sample DNA. To avoid non-specific binding, the PCR reactions were carried out in high-stringency touch-down conditions: 1 cycle of initial denaturation at 95 °C for 5 min. This was followed by 5 cycles of denaturation at 96 ºC for 20 s, annealing at 70 ºC for 45 s, and extension at 72 ºC for 25 s; then 21 cycles at 96 ºC for 25 s, 65 ºC for 50 s and 72 ºC for 30 s; and finally 6 cycles at 96 ºC for 30 s, 55 ºC for 60 s, and final extension was at 70 ºC for 2 min.

Ten (10) μl of the PCR products were run on 1.5% Tris-borate-EDTA/ ethidium bromide agarose gel (Simply, South Korea). The gel images were also documented in the questionnaire.

2.4. Statistical analysis

The frequency of genotypes and alleles compared between the general population and COVID-19 patients for significance, using χ2 (Fisher’s exact test). The odds ratio (OR) and 95% confidence intervals (95% CI) were used to verify the effects. Graphpad prism 9.1.1 software (GraphPad Software, San Diego, California USA) was used to perform statistical analysis. p-value less than 0.05 was considered as significant.

3. Results

The patients’ clinical information has been shown in Table 1. The data contained age groups from below 20 to over 70 years old. Out of 105 patients, 48 (45.71%) were male and 57 (54.29%) were female. CT scan performed for 31 patients included; moderate, 17 (16.19%) and severe 14 (13.33%). SpO2 values of 43 (40.95%) were lower than 93. Acute-phase reactant parameters like; CRP, ESR, D-dimer and serum ferritin were also documented. PCR positive test, CT scan and SPO2 were also documented in the questionnaire.

Three PCR reactions were needed for the genotyping of each DNA sample. The presence of a 173 bp band in PCR reactions (E2, E3 and E4) indicated the presence of a corresponding allele. The presence of a 406 bp band in every PCR reaction was necessary as it was indicated successful amplification produced by control primers. The absence of both allele-specific amplicon (173 bp) and control amplicon (406 bp) in any reaction is regarded as a failure.

The frequency of six different genotypes and three different Apo E alleles were calculated for COVID-19 patients and compared to the Apo E frequency data in a general population which we previously obtained in a separate study (Al-Jaf, 2021). All the three alleles E2, E3 and E4 have been found in both groups. However, E2/E2 and E4/E4 genotypes were not found in both healthy and patient groups, and E2/E4 genotype has not been found in the healthy group. The frequency results from both healthy controls and SARS-CoV-2 infected patients are summarized in Table 2.
In the current study, none of both homozygous E2/E2 and E4/E4 genotypes have been detected in the COVID-19 infected individuals, and this might be due to the low frequency of both Apo E2 and E4 alleles among the general population in this area, as it was suggested in our previous study (Al-Jaf, 2021). The frequency of both Apo E2/E3 and E3/ E4 genotypes was lower and that of Apo E3/E4 was higher in the patient group compared to the general population, however, none of these differences were statistically significant but the results were comparable. Regarding the Apo E alleles, the frequency of Apo E2 remained somewhat constant and the frequency of Apo E3 was lower in the patient group but it was not significant. These findings suggest the neutral role of Apo E2 and the possible protective role of the Apo E3 allele. Interestingly, the frequency of the Apo E4 allele among the COVID-19 patient group is more than twice its frequency compared to the general population and it was statistically significant. These data suggest that the Apo E4 allele might play a role in the COVID-19 infection. Previous studies have shown that the Apo E4 allele directly plays roles in inducing pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) while Apo E3 regulates these cytokines (Figuerola et al., 2018). It seemed that the increase in the frequency of the E4 allele is at the expense of a decrease in the E3 allele as its frequency decreased by the same ratio in COVID-19 infected individuals. This finding suggests the possible protective role of E3 and predisposing role of E4 alleles. Given that Apo E polymorphism is associated with most of the comorbidities related to COVID-19 infection like cardiovascular diseases, diabetes, hypercholesterolemia, and dementia. The effect of the E4 isoform could be indirect as it is predisposing to these comorbidities. However, a recent UK Biobank cohort study showed that the Apo E4 allele increases the risk of severe COVID-19 infection even after excluding the role of comorbidities like dementia, cardiovascular disease, and type-2 diabetes (Kuo et al., 2020).

The results obtained in this study are similar to the findings by Kuo and colleagues as they suggested a 2.2 fold increase in acquiring COVID-19 infection among Apo E4 homozygotes compared to Apo E3 homozygotes (Kuo et al., 2020a). However, other studies, utilizing UK Biobank data, have found that the role of Apo E4 was weakened after normalization of the results to associated comorbidities like AD, and CAS, suggesting the adding effect of these comorbidities to the role of Apo E4 allele (Kosmicki et al., 2020).

In another study performed among Czech subjects, Hubacek and colleagues found no significant difference in the frequency of Apo E alleles in 408 SARS CoV-2 PCR-positive subjects compared to the general population. However, when they compared symptomatic and asymptomatic COVID-19 subjects separately with the control population, they found a significant difference in the frequency of the Apo E4 allele between symptomatic patients and the control groups (Hubacek et al., 2021). Given that our COVID-19 subjects were symptomatic patients the results obtained by Hubacek and colleagues might not be in real contradiction to our results, as they found a significant difference when compared the symptomatic group of their COVID-19 subjects to the general population. In a cohort of 913 older volunteers in Spain, aged 75–90 years, similar results were achieved as it has been found that

### Table 1

| Parameters | Comorbidities | Demographic data |
|-----------|--------------|-----------------|
|            |              | Age groups No.  | Male No.  | Female No. |
|            |              | 1–20 years 6    | 2          | 4          |
|            |              | 21–30 years 11  | 5          | 6          |
|            |              | 31–40 years 21  | 9          | 12         |
|            |              | 41–50 years 19  | 9          | 10         |
|            |              | 51–60 years 13  | 7          | 6          |
|            |              | Over 60 years 35| 16         | 19         |

### Table 2

| Genotypes and alleles | General population N (%) N = 114 | SARS-CoV-2 Patients N (%) N = 105 | p-value | OR (95% CI) |
|-----------------------|----------------------------------|-----------------------------------|---------|-------------|
| E2/E2                 | 0 (0.0)                          | 0 (0.0)                           | 0.8277  | 0.8534      |
|                       | 14 (12.3)                        | 10 (9.5)                          |         | (0.3584 to 2.035) |
| E2/E4                 | 0 (0.0)                          | 3 (2.86)                          | 0.6820  | 0.9087      |
|                       | 92 (80.7)                        | 77 (73.33)                        |         | (0.6075 to 1.355) |
| E3/E4                 | 8 (7.0)                          | 15 (14.23)                        | 0.1180  | 2.240       |
|                       |                                  |                                   |         | (0.9004 to 5.389) |
| E4/E4                 | 0 (0.0)                          | 0 (0.0)                           | >0.9999 | 1.069       |
| E2                    | 14 (6.10)                        | 13 (12.19)                        | >0.9999 | (0.5021 to 2.226) |
| E3                    | 206 (90.40)                      | 179 (85.24)                       | 0.1243  | 0.8003      |
| E4                    | 8 (3.50)                         | 18 (8.57)                         | 0.0268<sup>+</sup> | 2.589 (1.092 to 5.746) |

N = Sample number, NA = Not applicable; because of zero values, OR = Odds ratio, CI = Confidence interval.
<sup>*</sup> Significant.

The statistical analysis of the data showed non-significant differences between the frequency of the six genotypes between the general population and COVID-19 patients, despite the relative difference in the frequency of E3/E4 genotype (P = 0.1180) between both groups. However, regarding the frequency of alleles, the Apo E4 allele is significantly increased (P = 0.0268) in COVID-19 patients compared to the general population. While the differences between Apo E2 and Apo E3 alleles were not significant comparing the two groups.

### 4. Discussion

Attempting to find the causes of heterogeneity in COVID-19 clinical manifestation, and severity, a large number of studies have been performed. The epidemiological studies identified several risk factors for developing severe disease. More recently, studies have focused on host genetic factors which lead to the identification of possible genetic variants that may be associated with disparities in COVID-19 disease outcome (Ellinghaus, 2020). ApoE protein is a multi-faceted protein that has been found associated with many diseases (Smith, 2000). Several studies have been performed to reveal a possible association between Apo E polymorphism and COVID-19 acquisition and severity (Kuo et al., 2020b); including few experimental studies (Hubacek et al., 2021) and the current study would be one of that few.
the Apo E4 allele was associated with the presence of symptoms and clinical severity of COVID-19 infection (Del Ser et al., 2021). A recent in vitro study also supported the harmful association of Apo E4 allele with the severity of COVID-19 infections. The study has found that neurons and astrocytes which expressed Apo E4 allele are more susceptible to SARS-CoV-2 infection than those expressed Apo E3, and more severe response are exhibited by Apo E4 carried astrocytes to SARS-CoV-2 infection than those which carried Apo E3 allele (Wang et al., 2021b). Thus, both in vitro and association studies support the Apo E4 allele plays detrimental roles in the clinical outcomes of COVID-19 patients.

Revealing genetic predisposition to COVID-19 infection is important for identifying high-risk individuals for the purpose of health care and preventive measure prioritizations like vaccination and monoclonal antibody treatment, it is also important for the development of drugs as it might guide the treatment targets, in the similar way that CR5 variation and HIV infection, helped in identifying drug targets and therapeutic strategies (Kosmicki et al., 2020). The limitation of this study was that the sample numbers were not enough to perform statistical analyses between comorbid and non-comorbid patients to observe any roles of comorbidities in the severity of the disease.

In conclusion, this work found an association of Apo E4 allele in COVID-19 patients compared with the general population in a cohort study in Iraq for the first time. Also, Apo E3 was suggested to have protective roles; however, larger samples are required for future study to confirm this suggestion. There were no associations in the Apo E2 allele between both patient and general population groups. Future work should also focus on various ethnic backgrounds and comorbidities using a large number of samples to see whether this association can be generalized to the entire world.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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