ORIGINAL ARTICLE

Human Leukocyte Antigen G 14 Base Pair Insertion/Deletion Polymorphism and COVID-19 Susceptibility and Severity in Egyptian Patients: A Case Control Study

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ABSTRACT

Background: *HLA-G inhibits the immune system and has been linked to the risk and prognosis of numerous infectious diseases.* ***Objective:*** *to investigate the impact of HLA-G 14 bp insertion/deletion polymorphism on the susceptibility and severity of COVID-19.* ***Methodology:*** *This was a case-control study that comprised a total of 86 participants divided into two equal groups: 43 healthy controls and 43 COVID-19 Egyptian patients. Determination of the genetic polymorphism was performed for all study participants.* ***Results:*** *We found a statistically significant difference between both cases and healthy controls as regards the distribution of HLA-G genotypes (p-value=0.018). Healthy controls were 5.2 times more likely to have the INS/DEL genotype (OR= 5.2; 95% CI: 1.57-17.18) (p-value=0.007) and 2.7 times to have the INS/INS genotype (OR= 2.76; 95% CI: 0.76-9.96 (p-value=0.11). Although we found an association between HLA-G polymorphism and COVID-19 severity (p-value=0.002), however, it was not confirmed by logistic regression. The presence of the INS/INS genotype was linked to higher odds of developing severe COVID-19 (OR=3.66; 95% CI: 0.173-77.5) and (OR= 6.28; 95% CI: 0.577- 68.4) in comparison to the DEL/DEL and INS/DEL genotypes, respectively, but these higher odds were not statistically significant (p-value=0.40 and 0.13), respectively.* ***Conclusion:*** *Among healthy controls, the most common genotype was INS/DEL. The DEL/DEL genotype was associated with increased susceptibility to COVID-19 infection. HLA-G polymorphisms do not affect the severity of COVID-19.*

INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a newly discovered enveloped RNA beta coronavirus that is responsible for Coronavirus Disease-2019 (COVID-19)[1]. This unique infection might be asymptomatic or progress to severe pneumonitis and Acute Respiratory Distress Syndrome (ARDS). Many clinical risk factors for a severe clinical course have been identified including age and the existence of co-morbidities[2]. However, the clinical progression of the disease is also influenced by host variables such as the host's immune response[3].

In response to a viral infection, effective innate and adaptive immune cells arise spontaneously. Natural killer (NK) cells are innate immune cells that are responsible for the elimination of pathogens and virus-infected cells, as well as the prevention of viral spread. Virus-specific CD8+ cytotoxic T cells are also activated, causing virus-infected cells to die and virus-specific CD4+ T cells to activate[4].

Human leukocyte antigen G (HLA-G) is capable of suppressing both innate and adaptive immune responses and switching cytokine production from Th1 cytokines to Th2 cytokines, leading to establishment of an immunologically tolerant microenvironment. Human viruses increase the expression of HLA-G on the surface of infected cells. As a result, they can multiply and spread without eliciting significant host immune responses [5].

HLA-G 14 base-pairs (bp) insertion (INS)/deletion (DEL) polymorphism located in the 3′ untranslated region (UTR) of exon eight has been demonstrated to alter HLA-G mRNA stability and expression[6]. Therefore, we conducted this study to investigate the distribution of HLA-G 14 bp INS/DEL polymorphism in Egyptian COVID-19 patients versus healthy controls and to assess the possible association between this polymorphism and the severity of COVID-19.

METHODOLOGY

Study setting & design:

This case-control study was conducted at Zagazig University Hospitals, Sharqia Governorate, Egypt, in May 2020. The study included all COVID-19 patients

that were admitted to the quarantine hospital during the study period (comprehensive sample, n=43). The study also comprised (n=43) healthy controls with no respiratory symptoms for the past 12 months. All the participants were of Egyptian ethnicity.

Study population:

The patients were eligible to be enrolled in the study if they met the following inclusion criteria: age ≥ 18 years, males or females, and confirmed diagnosis of COVID-19. The diagnosis of COVID-19 was based on the clinical, laboratory, and radiological data, including SARS-CoV-2 Real-Time Polymerase Chain Reaction (RT-PCR) from nasopharyngeal swabs and non-contrast chest Computed Tomography (CT). The exclusion criteria for both cases and controls included: age < 18 years or pregnant females.

Patients were classified as having mild, moderate, or severe disease using the criteria laid out in the Egyptian Ministry of Health management plan for COVID-19 [7]. The mild COVID-19 definition was applied for those patients who showed just clinical symptoms, leucopenia, or lymphopenia but no radiological evidence of pneumonia. Patients who had radiographic evidence of pneumonia in addition to clinical symptoms and/or leucopenia or lymphopenia were considered to have a moderate COVID-19. The diagnosis of severe cases required the presence of at least one of the following conditions:"(1) respiratory distress with a respiratory rate >30/min; (2) resting blood oxygen saturation < 92%; or (3) a partial pressure of arterial blood oxygen (PaO2)/oxygen concentration (FiO2) < 300 mmHg. Critically ill patients include those who have experienced respiratory failure requiring mechanical ventilation, shock, or failure of any other major organ".

Genotyping of HLA-G polymorphism:

Under sterile conditions, 2 mL of EDTA anti-coagulated venous blood was extracted from each participant. Following the manufacturer's guidance, genomic DNA was extracted using genomic DNA purification kits (Gene JET, Thermo Scientific, USA). After DNA extraction, the samples were labeled and stored at -20°C. According to Alegre et al.[8], genotyping of 14 bp INS/DEL polymorphism in the 3' UTR of the HLA-G gene using RT-PCR of exon 8 was accomplished. In short, "100 ng of genomic DNA was amplified in a 25 µL reaction containing the following reagents (iNtRON Biotechnology, Korea): 1x Reaction Buffer, 2.5 mm of each dNTP, 1.5 mm $MgCl_2$, 2.5 U Taq Polymerase, and 10 pmol of forward (5'-GTGATGGGCTGTTTAAAGTGTC ACC-3') and reverse (5'-GGAAGGAATGCAGTTCAGCATGA-3') primers". The thermal cycler (Biometra) was set to the following parameters: "95°C for (180 seconds), followed by 35 cycles of denaturation at 95°C for (60 seconds), annealing at 64°C for (60 seconds), and elongation at 72°C for (60 seconds), with the final elongation at 72°C for (600 seconds)". The amplified PCR products were electrophoresed in a 3% agarose gel containing ethidium bromide (0.5 g/ml) (Sigma, USA) for 40 minutes and then visualized under UV light with a gel documentation system. Two distinct observers interpreted the gene polymorphism. Depending on the 14bp deletion of exon 8, the amplified PCR products were either 224 or 210 bp in length or both 224 and 210 bp.

Ethical approval:

The study was approved by the Ethics Committee of the Faculty of Medicine, Zagazig University (ZU-IRB No.6433). All participants or their relatives signed an informed written consent.

Statistical analysis:

Data were analyzed using SPSS version 26 (New York; IBM Corp.). Data distribution was tested using the Kolmogorov–Smirnov test. Median and range were used to summarize numerical variables, while frequency and percentages were used to summarize categorical variables. The Mann-Whitney-U test was used to compare numerical variables between two groups; the Kruskal-Wallis test was used to compare numerical variables for more than two groups. The Chi-square or Fisher's exact test, as appropriate, was used to compare categorical variables. Logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI). P-value ≤0.05 was considered significant.

RESULTS

Characteristics of the patients:

The characteristics of COVID-19 patients included in the study are shown in table (1). The study comprised (n=25, 58.1%) females and (n=18, 41.9%) males. The median age of patients was 56 (range: 27-80). All enrolled patients were classified into three groups based on COVID-19 severity; mild (n=6), moderate (n=16), and severe (n=21). In terms of age and gender, there was no significant difference across patient sub-groupings. However, there was a significant difference between the sub-groups as regards the clinical symptoms (the presence of dyspnea, ARDS, and acute cardiac injury).The laboratory investigations across patient sub-groupings are shown in table (2).

Table 1: Demographic and clinical characteristics of the included patients

Variables	Total (N=43)	Mild (N=6)	Moderate (N=16)	Severe (N=21)	P-value
Age, years (median, range)	56 (27-80)	46 (29-56)	57 (30-80)	61 (27-75)	0.126
Sex (N, %)					
Male	18 (41.9%)	3 (50%)	5 (31.2%)	10 (47.6%)	0.629
Female	25 (58.1%)	3 (50%)	11 (68.8%)	11 (52.4%)	
Clinical symptoms (N, %)					
Cough	36 (83.7%)	3 (50%)	14 (87.5%)	19(90.5%)	0.077
Dyspnea	37 (86%)	3 (50%)	13 (81.3%)	21(100%)	**0.005***
Myalgia	38 (88.4%)	5 (83.3%)	13 (81.3%)	20 (95.2%)	0.323
Sputum production	16 (37.2%)	1 (16.7%)	6 (37.5%)	9 (42.9%)	0.632
Chest pain	26 (60.5%)	1 (16.7%)	10 (62.5%)	15 (71.4%)	0.066
Sore throat	16 (37.2%)	1 (16.7%)	7 (43.8%)	8 (38.1%)	0.569
Rhinorrhea	13 (30.2%)	0 (0%)	7 (43.8%)	6 (28.6%)	0.180
Vomiting	17 (39.5%)	1 (16.7%)	8 (50%)	8 (38.1%)	0.435
Diarrhea	17 (39.5%)	1 (16.7%)	8 (50.0%)	8 (38.1%)	0.435
Nausea	19 (44.2%)	2 (33.3%)	8 (50.0%)	9 (42.9%)	0.840
ARDS	12 (27.9%)	0 (0%)	0 (0%)	12 (57.1%)	**<0.001***
Headache	26 (60.5%)	1 (16.7%)	11 (68.8%)	14 (66.7%)	0.087
Presence of Co-morbidities	28 (65.1%)	3 (50%)	10 (62.5%)	15 (71.4%)	0.627
Acute cardiac injury	6 (14%)	0 (0%)	0 (0%)	6 (28.6%)	**0.035***

** P value is statistically significant.*

Table 2: Laboratory characteristics of the included patients

Variables	Total (N=43)	Mild (N=6)	Moderate (N=16)	Sever (N=21)	P-value
CRP (mg/L)	77 (4.2-786)	9.6 (4.2-24)	42.5 (6.5-257)	137 (12-786)	**<0.001***
D-Dimer (mg/L)	0.7 (0.1-8.1)	0.3 (0.1-0.7)	0.5 (0.1-2)	1.3 (0.2-8.1)	**<0.001***
LDH (U/L)	352 (90-983)	213.5 (90-369)	220 (130-595)	560 (256-983)	**<0.001***
CK (U/L)	1.2 (0.02-7.6)	0.4 (0.04-1.3)	0.8 (0.02-2.3)	2.4 (0.3-7.6)	**0.001***
PT (seconds)	14.3 (10.8-23.4)	14 (13.4-15.6)	14 (10.8-20.3)	14.7 (11.2-23.4)	0.601
ALT (IU/L)	28 (10-169)	17.5 (14-28)	29.5 (14-65)	35 (10-169)	**0.014***
AST (IU/L)	33 (13-320)	18 (15-26)	33 (13-114)	39 (17-320)	**0.002***
Ferritin (µg/L)	474 (23.3-3293)	64 (23.3-116)	337 (155-1956)	776 (299-3293)	**<0.001***
ESR (mm/hour)	65 (20-140)	25 (20-75)	36.5 (23-105)	80 (25-140)	**<0.001***
Procalcitonin (ng/ml)	0.2 (0.01-13)	0.04 (0.01-3)	0.1 (0.02-9)	2.3 (0.01-13)	**<0.001***
Troponin (ng/ml)	3.8 (0.3-12.8)	1.7 (0.3-6.7)	2.9 (0.3-6.7)	8.6 (1.3-12.8)	**<0.001***
Creatinine (mg/dl)	0.8 (0.4-13.2)	0.5 (0.4-0.9)	0.8 (0.4-2.4)	1 (0.6-13.2)	**0.005***
WBCs count (10^3/µL)	9.2 (0.7-24)	6.8 (0.7-22.1)	5.2 (4-8.6)	13.1 (4.1-24)	**0.004***
Lymphocyte (10^3/µL)	1.4 (0.2-7)	1.8 (0.2-4)	1.3 (0.4-7)	1.4 (0.6-4)	0.883
Platelets (10^3/µL)	235 (67-455)	234 (328-392)	281 (67-432)	206 (95-455)	0.365

****P value is statistically significant; the values are for median (range). Abbreviations: ALT:** Alanine transaminase; **AST:** Aspartate transaminase; **CK:** Creatinine Kinase; **CRP:** C-Reactive Protein**; ESR:** Erythrocyte Sedimentation Rate**; LDH:** Lactate Dehydrogenase**; PT:** Prothrombin Time.*

Characteristics of the healthy controls:

The socio-demographic characteristics of healthy controls are shown in table (3). The median age for the control group was 35 (range: 24-80). Males accounted for (n=16; 37.2%). In terms of age and gender there were statistically significant differences between patients and controls (p-value <0.001 and 0.05), respectively.

Distribution of HLA-G polymorphism among both patients and healthy controls:

As demonstrated in table (3), there was a statistically significant difference in the distribution of HLA-G genotypes among COVID-19 patients and healthy controls (p value=0.018). Healthy controls were 5.2 times more likely to have the INS/DEL genotype (OR= 5.2; 95% CI: 1.57-17.18) (p-value=0.007). In terms of the INS/INS genotype, although the healthy controls were 2.7 times more likely to have this genotype, when using the DEL/DEL genotype as a reference, (OR= 2.76; 95% CI: 0.76-9.96), however, this difference was not statistically significant (p value=0.11).

Table 3: Comparison between COVID-19 patients and healthy controls as regards age, sex, and distribution of the HLA-G polymorphism

Variables	Case (N=43)	Control (N=43)	P-value
Age, years (median, range)	56 (27-80)	35 (24-80)	**<0.001***
Sex (N, %)			
Male	18 (41.9%)	16 (37.2%)	**0.052***
Female	25 (58.1%)	27 (62.8%)	
HLA 14 bp polymorphism (N, %)			
DEL/DEL	15 (34.9%)	5 (11.6%)	
INS/DEL	15 (34.9%)	26 (60.5%)	**0.018***
INS/INS	13 (30.2%)	12 (27.9%)	
HLA-G 14 bp polymorphism	**OR**	**(95% CI)**	**P-value**
DEL/DEL	Reference	Reference	Reference
INS/DEL	5.2	(1.57-17.18)	**0.007***
INS/INS	2.7	(0.76-9.96)	**0.11**

** P value is statistically significant.*

HLA-G polymorphism as predictor of COVID-19 severity:

An association between HLA-G polymorphism and COVID-19 severity (p-value=0.002) was found as shown in table (4), however, this significance was not confirmed by logistic regression. According to the univariate logistic regression, the presence of INS/INS genotype was linked to higher odds of developing severe COVID-19 (OR=3.66; 95% CI: 0.173-77.5) and (OR= 6.28; 95% CI: 0.577-68.4) in comparison to the DEL/DEL and INS/DEL genotypes, respectively. However, these higher odds were not statistically significant (p-value=0.40 and 0.13), respectively as shown in table (5).

Table 4: Association between HLA-G polymorphism and COVID-19 severity

Variables	DEL/DEL	INS/DEL	INS/INS	P value
Mild cases (n=6)	1 (16.7%)	4 (66.6%)	1 (16.7%)	**0.002***
Moderate cases (n=16)	11 (68.7%)	4 (25%)	1 (6.3%)	
Severe cases (n=21)	3 (14.3%)	7 (33.3%)	11 (52.4%)	
Total (n=43)	15	15	13	

** P value is statistically significant.*

Table 5: Univariate logistic regression for predictors of COVID-19 severity

Variable	OR (95%CI)	P value
Age	1 (0.98-1.1)	0.3
Gender	0.63 (0.19-2.13)	0.5
Presence of Co-morbidities	1.73 (0.48-6.18)	0.4
HLA-G INS/DEL (vs. DEL/DEL)	0.58 (0.04-7.66)	0.68
HLA-G INS/INS (vs. DEL/DEL)	3.66 (0.173-77.5)	0.40
HLA-G INS/INS (vs. INS/DEL)	6.28 (0.577-68.4)	0.13

DISCUSSION

The spread of the COVID-19 pandemic poses a serious threat to public health. An effective host's antiviral immune response is crucial for virus elimination and disease management. However, depletion of the host's cellular immune response as well as a reduction in the number of immune cells is noted in COVID-19 patients, which is followed by a bad prognosis [9]. HLA-G binds to multiple immune inhibitory receptors, leading to immunosuppression and viral escape [10]. Several studies have revealed the up-regulation of HLA-G during numerous viral infections, including herpes simplex virus type-1 (HSV-1), human cytomegalovirus (CMV), human immune-deficiency virus (HIV-1), hepatitis B virus (HBV), hepatitis C virus (HCV), rabies, and influenza A virus [11-16].

This was the first study to compare HLA-G 14bp polymorphism in Egyptian COVID-19 patients versus healthy controls and to explore its association with the disease severity. The current study revealed that INS/DEL was the most prevalent genotype among healthy controls, while the DEL/DEL genotype was associated with increased an incidence of COVID-19 infection. The HLA-G 14 bp polymorphism could not predict COVID-19 severity. Only one study investigated the same genetic variant in COVID-19 patients and

healthy control was available in the literature to compare with. Consistent with our findings, they couldn't verify that the HLA-G 14bp polymorphism could influence COVID-19 severity and similarly reported that DEL/DEL genotype enhances COVID-19 susceptibility in Iraqi persons[17].

Interestingly, a genome-wide association analysis conducted on critically ill COVID-19 patients found a link between a mutation of the HLA-G gene known as rs9380142 and the susceptibility to COVID-19 infection. It is important to note that this variant is found in exon eight of the HLA-G gene, near to the position of 14bp INS/DEL polymorphism. This finding may imply that certain HLA-G genetic variations are associated with an increased risk of COVID-19 infection [18]. In addition, Rizzo et al. observed the expression of HLA-G on the intestinal epithelial cells of a patient who had recovered from COVID-19 [19]; this is the same place that correlates to the entry of SARS-CoV-2.

In contrast, Lv et al. carried out a meta-analysis to investigate whether or not the HLA-G 14 bp polymorphism is a risk factor that increases the likelihood of contracting a viral infection. They concluded that it may not play a role in the susceptibility to viral infection[20]. Furthermore, Zhang et al., contrary to our findings, reported an association between over-expression of HLA-G and COVID-19 severity [21]. Remarkably, The HLA-G polymorphism' impact on the serum soluble HLA-G levels is controversial [22, 23].

Implications of the study:

This work shed light on the link between HLA-G 14 bp INS/DEL polymorphism and the susceptibility and outcome of COVID-19. To the best of our knowledge, this was the first research investigating this genetic polymorphism in Egyptian COVID-19 patients. Understanding how genes are liked with COVID-19 could explain why certain individuals are more susceptible to infection or developing severe disease and might suggest particular targets for personalized therapy.

Limitations:

This study was limited by the relatively small sample size. Future studies with larger sample sizes are required.

CONCLUSION

HLA-G 14 bp INS/DEL polymorphism has no influence on COVID-19 severity. The INS/DEL genotype was the most common among the healthy controls, while the DEL/DEL genotype was associated with higher susceptibility to COVID-19 infection.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

Authors' Contribution:

Dina M. Ali is a principal investigator responsible for the concept, study design, data analysis and interpretation, writing the draft, and critical revision of the final manuscript. Alia A. El Shahawy is a principal investigator responsible for the concept and acquisition of data. Nagwan A. Ismail was attributed to acquisition of data.

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