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Research article

# Evaluation the frequencies of HLA alleles in moderate and severe COVID-19 patients in Iran: A molecular HLA typing study

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ABSTRACT

*Background:* Severe acute respiratory syndrome coronavirus 2 was first reported in December 2019 and it has spread globally ever since. The HLA system is crucial in directing anti-viral immunity and recent studies are investigating the possible involvement of the HLA genes on the severity of immune inflammation in different phases of COVID-19.
*Methods:* In this cross-sectional study, peripheral blood-extracted genomic DNAs of 109 COVID-19 patients and 70 healthy controls were genotyped for different alleles of HLA-A, HLA-B, and HLA-DRB1 loci using sequence-specific primer PCR method.
*Results:* The results indicated that frequencies of HLA-DRB1*11:01 and HLA-DRB1*04:03 were significantly higher in severe patients rather than moderates (p: <0.001 and 0.004, respectively). Also, it was observed that HLA-DRB1*04:01 was more frequent in moderate patients and healthy controls (p:0.002). In addition, HLA-B*07:35, and HLA-DRB1*07:01 showed higher frequencies in patients compared with controls (p: 0.031 and 0.003 respectively). Inversely, due to the higher frequencies of HLA-B*51:01 (p:0.027), HLA-DRB1*11:05 (p:0.003), HLA-DRB1*13:05 (p:0.022), and HLA-DRB1*14:01 (p:0.006) in healthy individuals rather than patients, they may be associated with COVID-19 resistance.

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*Conclusion:* The results show that, based on the population differences, the type of alleles related to the severity of COVID-19 is different, which should be clarified by designing large-scale studies in order to develop HLA-based treatments and vaccines.

## 1. Introduction

Severe acute respiratory syndrome coronavirus 2, a recently identified strain of beta coronaviruses, has affected more than 697 million people worldwide from December 2019 until November 2023 [1]. SARS-CoV-2 has emerged as an international public health emergency with a relatively high mortality rate of 3.4% [2]. It is reported that 18–30% of the infected people may be affected without any clinical signs, while others may show mild, moderate, severe, or critical symptoms [3]. Although many studies are conducted to evaluate the genetic features of SARS-CoV-2, HLA association and immunopathogenesis, various aspects such as patients' susceptibility, disease severity, and clinical outcomes need to be more clarified [4–11]. Investigating the effects of HLA alleles' distribution on vulnerability or resistance to SARS-CoV-2 is important that may help for better diagnosis and management in different geographical regions [12]. HLA molecules, which are encoded by major histocompatibility complex genes, are highly polymorphic, and involved in the regulation of the immune response [13]. Genetic diversity of HLA alleles in various populations may influence COVID-19 severity [14–16]. Population differences may be closely associated with the inconsistent results of HLA association with SARS-CoV-2 infection severity [17–20]. Overall, given the high susceptibility and mortality rates among patients in Iran [21], the current study was conducted to find the probable relation between the frequencies of HLA alleles and the clinicopathological manifestations of COVID-19 among hospitalized patients. Here, we reported our observations based on sequence-specific primer PCR prepared from blood samples of 109 Iranian COVID-19 patients and 70 healthy adults as control, with the aim of identifying the alleles that are responsible for a higher susceptibility and severity of the disease. In fact, the specific purpose of this study was to investigate the presence or absence of the most common alleles associated with HLA class I and HLA class II in Iranian patients with moderate and severe COVID-19 and comparing them with healthy subjects. In addition, all three studied groups were compared and statistically analyzed in terms of possible differences in the frequency of alleles found.

## 2. Materials and methods

### 2.1. Case selection

A number of 109 hospitalized patients and 70 healthy subjects were enrolled in this study. All participants signed the informed consent form before joining the project. The patients were initially admitted from May 25 to August 1, 2020. We screened all clients who had a fever, cough, and radiographic presentation at the initial assessment or close contact with an infected individual within the past 14 days. The diagnosis of COVID-19 was based on a SARS-CoV-2 (alpha/Wuhan variant) nucleic acid detection kit (PCR-Fluorescence Probing) according to the manufacturer's protocol (Sansure Biotech Inc. China) for detecting SARS-CoV-2 RNA in the throat or upper nasopharyngeal swab samples. According to the Infectious Diseases Society of America (IDSA) guidelines and considering the state of the disease, patients were subdivided into two groups including 57 moderate and 52 severe cases and all were admitted to the hospital. The moderate cases included adults with signs of pneumonia (i.e., cough, fever, fast breathing, and dyspnea), $O_2$ saturation $\geq$90% and $\leq$93%, respiratory involvement (unilateral lung involvement), and PCR test positivity for SARS-CoV-2. However, the inclusion criteria for severe patients were $O_2$ saturation $<$90%, severe pneumonia, bilateral respiratory involvement, PCR positive report, and respiratory rate $>$30 breaths per minute [22]. Critical ICU-admitted patients were not included in this study. non-participation of critical ICU-admitted patients in this study is considered as one of the limitations. Patients were classified in such a way that we considered $O_2$ saturation below 90% as severe, but when it was corrected by using reservoir bags, we did not admit the patient to the ICU. Another point is that due to the overload of the patients and the peak incidence of disease at the time of sampling, as well as the limited capacity of the ICU, we were very careful in selecting critical patients. Therefore, the patients who used oxygen with nasal canula and its reinforcement with reservoir bags were considered as severe, and if the O2 saturation level was not corrected with

**Table 1**
The ordered treatments for patients based on the national COVID-19 therapeutic guidelines at the sampling time. The dosage and type of the drugs varied for each group according to the severity of disease.

| | Drug | Dosage | Time |
|---|---|---|---|
| 1 | Hydroxychloroquine Sulfate | 200 mg tablets | First day: 400 mg BD |
| 2 | Chloroquine phosphate | 250 mg tablets | First day: 500 mg BD |
| 3 | Kaletra (Lopinavir, Ritonavir tablets) | 200/50 mg | Second day: 2 tablets BD up to 14 days |
| 4 | Atazanavir/Ritonavir tablets | 300/100 mg | Second day: 2 tablets BD up to 14 days |
| 5 | [a]Vitamin D | 1000 IU | Daily/PO |
| 6 | [a]MgSO4 | 250 mg | BD/PO |
| 7 | [a]Vitamin C | 500–1000 mg | Daily/PO |
| 8 | [a]Famotidine | 40 mg | Daily/PO |

[a] Supportive treatments.

reservoir bags, the patients were considered as critical ICU-admitted, where NIV (non-invasive mechanical ventilation) mask and intubation connected to the ventilator were used. The ordered treatments for the patients are presented in Table 1. Moreover, 70 healthy subjects aged between 30 and 60 years with no history of underlying diseases or COVID-19 positivity were selected as the control group. The healthy control subjects were selected from the people who did not show any clinical signs related to SARS-CoV-2 infection despite being exposed to COVID-19 patients at work or at home. Absence of any clinical symptoms, normal oxygen saturation level, negativity of PCR and serologic tests, and normal chest CT scan were all confirmed in these individuals as inclusion criteria. Anti-SARS-CoV-2 IgG ELISA kit (Dia. Pro Diagnostics - Milano, Italy) and anti-IgM COVID-19 ELISA kit (Euro-immune - Schleswig-Holstein, Germany) were used for serologic testing. Also, nucleic acid detection kit (Sansure Biotech Inc. China) was applied for detecting SARS-CoV-2 RNA in the throat or upper nasopharyngeal swab samples. Also, after asymptomatic phase, no seroconversion was observed in any of these individuals.

### 2.2. Blood samples

Genomic DNAs were obtained from the blood samples of the participants all of whom gave informed consent and followed the Declaration of Helsinki. Moreover, the Ethical Committee of Ahvaz Jundishapur University of Medical Sciences approved the project (No. IR.AJUMS.REC.1399.142).

### 2.3. DNA extraction and sequence specific primer PCR

DNA was extracted from blood samples using a MagCore® HF 16 Plus automated nucleic acid extractor (RBC Bioscience, Taiwan), which has the specific kit with the highest efficiency for each type of extraction. Automated DNA extraction followed the protocol that was preinstalled on the extractor, according to the manufacturer's instructions. Then, the quality of extracted DNA was examined using Nanodrop. The samples were typed for the frequencies of HLA allele using the SSP PCR method (BAG Diagnostics - HLA SSP typing kit, Hessen, Germany). Based on the HLA typing kit, various alleles of HLA-A, HLA-B, and HLA-DRB1 were assessed in the present study. Given the higher frequency of HLA-DRB1, we evaluated more digits and determined the subtypes of HLA-DRB1. The Specific PCR amplification reaction was performed in 30 cycles according to the manufacturer's protocol. Amplicons were run on a 2% agarose gel and electrophoresis was carried out. Finally, Double-stranded DNA fragments were visualized with DNA-safe stain (Sinaclon Company, Iran).

**Table 2**
Demographic and laboratory findings of the enrolled patients. The age and sex profile of the control group are also given in the table. The statistical values are calculated using the Fisher's exact test.

| Variable | Moderate Patients | Severe Patients | P-value | Control group |
|---|---|---|---|---|
| Gender | | | | |
| Male, N (%) | 36 (63.2%) | 32 (61.5%) | 0.862 | 39 (55.7%) |
| Female, N (%) | 21(36.8%) | 20 (38.5%) | | 31 (44.3%) |
| Total, N (%) | 57 (52.3%) | 52 (47.7%) | | 70 |
| Age | | | | |
| <40 | 11 (19.3%) | 5 (9.6%) | 0.269 | 37 (52.8%) |
| 40–60 | 16 (28.1%) | 20 (38.5%) | | 33 (47.2%) |
| >60 | 30 (52.6%) | 27 (51.9%) | | |
| Laboratory Findings (mean ± SD) | | | | **P-value** |
| RR (bpm) | 25.03 ± 4.99 | 29.88 ± 5.78 | | **< 0.001**** |
| PR (bpm) | 84.21 ± 11.13 | 88.73 ± 13.39 | | 0.054 |
| $O_2$ saturation (%) | 94.84 ± 6.80 | 86.06 ± 11.90 | | **< 0.001**** |
| ESR (mm/hr) | 45.90 ± 23.74 | 62.42 ± 31.50 | | **0.004**** |
| Cr (mg/dL) | 1.65 ± 1.86 | 1.66 ± 1.26 | | 0.982 |
| AST (U/L) | 41.21 ± 21.82 | 75.78 ± 74.89 | | **0.003**** |
| BUN (mg/dL) | 24.57 ± 23.10 | 36.08 ± 30.86 | | **0.035*** |
| ALT (U/L) | 30.89 ± 26.79 | 48.48 ± 51.79 | | **0.038*** |
| ALP (U/L) | 198.19 ± 86.19 | 215.89 ± 103.64 | | 0.351 |
| Alb (g/dL) | 4.05 ± 0.74 | 4.10 ± 0.57 | | 0.671 |
| CRP (mg/L) | 40.1 ± 18.9 | 49.2 ± 21.3 | | **0.018*** |
| LDH (U/L) | 567.5 ± 229.1 | 802.4 ± 407.4 | | **< 0.001**** |
| D-dimer (ng/ml) | 564.1 ± 265.8 | 896.6 ± 476.3 | | **< 0.001**** |

Data are presented as mean ± SD or the frequency of individuals (percentage). According to the calculated values, levels of AST, BUN, ALT, CRP, LDH, ESR, $O_2$ saturation, respiratory rate and D-dimer are significantly different in severe patients as compared with moderates due to the severity of disease. Abbreviations. RR: Respiratory rate. PR: Pulse rate. ESR: Erythrocyte sedimentation rate. Cr: Creatinine. AST: Aspartate aminotransferase. ALT: Alanine aminotransferase. BUN: Blood urea nitrogen. ALP: Alkaline phosphatase. Alb: Albumin. CRP: C-reactive protein. LDH: Lactate dehydrogenase. D-dimer: Fibrine degradation products (FDP).

**Table 3**
Association between HLA alleles and COVID-19 incidence and severity in Iranian patients. DNA genotyping for severe/moderate patients and healthy subjects were performed and the frequencies of different alleles are analyzed. The table is subdivided into more frequent and less frequent alleles. If the total frequency of an allele in the patient group was less than 5% it was included in the less frequent alleles section.

More frequent alleles

| | Severe patients | | Moderate patients | | | Total patients | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Allele | N | F% | N | F% | P | N | F% | N | F% | P |
| A*01:01 | 17 | 32.7 | 12 | 21.1 | 0.201 | 29 | 26.6 | 19 | 27.1 | 1.000 |
| A*02:01 | 15 | 28.3 | 25 | 42.4 | 0.167 | 40 | 36.7 | 26 | 37.1 | 1.000 |
| A*03:01 | 10 | 19.2 | 10 | 18.0 | 1.000 | 20 | 18.3 | 16 | 22.9 | 0.567 |
| A*11:01 | 8 | 15.4 | 9 | 15.8 | 1.000 | 17 | 15.6 | 11 | 15.7 | 1.000 |
| A*24:02 | 16 | 30.8 | 14 | 24.6 | 0.523 | 30 | 27.5 | 19 | 27.1 | 1.000 |
| A*26:01 | 6 | 11.5 | 2 | 3.5 | 0.148 | 8 | 7.3 | 3 | 4.3 | 0.532 |
| A*30:01 | 4 | 7.7 | 10 | 17.5 | 0.157 | 14 | 12.8 | 6 | 8.6 | 0.470 |
| A*31:01 | 3 | 5.8 | 4 | 7.0 | 1.000 | 7 | 6.4 | 0 | 0.0 | 0.044 |
| A*32:01 | 7 | 13.5 | 3 | 5.3 | 0.189 | 10 | 9.2 | 11 | 15.7 | 0.235 |
| A*33:01 | 6 | 11.5 | 6 | 10.3 | 1.000 | 12 | 11 | 7 | 10 | 1.000 |
| A*68:01 | 7 | 13.5 | 9 | 15.8 | 0.792 | 16 | 14.7 | 5 | 7.1 | 0.156 |
| B*07:02 | 3 | 5.8 | 3 | 5.3 | 1.000 | 6 | 5.5 | 3 | 4.3 | 1.000 |
| B*07:35 | 27 | 51.9 | 27 | 47.7 | 0.564 | 54 | 49.5 | 23 | 32.9 | 0.031* |
| B*08:01 | 8 | 15.4 | 6 | 10.5 | 0.570 | 14 | 12.8 | 6 | 8.6 | 0.470 |
| B*14:02 | 5 | 9.6 | 1 | 1.8 | 0.101 | 6 | 5.5 | 4 | 5.7 | 1.000 |
| B*15:17 | 4 | 7.7 | 4 | 7.7 | 1.000 | 8 | 7.3 | 2 | 2.9 | 0.320 |
| B*18:01 | 7 | 13.5 | 4 | 7.0 | 0.346 | 11 | 10.1 | 10 | 14.3 | 0.477 |
| B*35:01 | 11 | 21.2 | 15 | 26.3 | 0.654 | 26 | 23.9 | 15 | 21.4 | 0.856 |
| B*35:02 | 6 | 11.5 | 5 | 8.8 | 0.754 | 11 | 10.1 | 2 | 2.9 | 0.082 |
| B*38:01 | 3 | 5.8 | 4 | 7.0 | 1.000 | **7** | 6.4 | 5 | 5.7 | 1.000 |
| B*41:01 | 4 | 7.7 | 9 | 15.8 | 0.244 | 13 | 11.9 | 8 | 11.4 | 1.000 |
| B*50:01 | 7 | 13.5 | 5 | 8.8 | 0.545 | 12 | 11.0 | 10 | 14.3 | 0.642 |
| B*51:01 | 3 | 5.8 | 4 | 7.0 | 1.000 | 7 | 6.4 | 12 | 17.1 | 0.027* |
| B*51:05 | 9 | 17.3 | 12 | 21.1 | 0.637 | 21 | 19.3 | 12 | 17.1 | 0.844 |
| B*52:01 | 4 | 7.7 | 6 | 10.5 | 0.745 | 10 | 9.2 | 6 | 8.6 | 1.000 |
| DRB1*01:01 | 5 | 9.6 | 6 | 10.5 | 1.000 | 11 | 10.1 | 5 | 7.1 | 0.598 |
| DRB1*03:01 | 11 | 21.2 | 15 | 26.3 | 0.654 | 26 | 23.9 | 28 | 40.0 | 0.030* |
| DRB1*04:01 | 1 | 1.9 | 12 | 21.1 | **0.002**** | 13 | 11.9 | 22 | 31.4 | 0.002** |
| DRB1*04:02 | 2 | 3.8 | 7 | 12.3 | 0.165 | 9 | 8.3 | 0 | 0.00 | 0.013* |
| DRB1*04:03 | 7 | 13.5 | 0 | 0.0 | **0.004**** | 7 | 6.4 | 0 | 0.0 | 0.044* |
| DRB1*07:01 | 15 | 28.8 | 14 | 24.6 | 0.668 | 29 | 26.6 | 6 | 8.6 | 0.003** |
| DRB1*11:01 | 26 | 50.0 | 10 | 17.5 | **< 0.001***** | 36 | 33.0 | 31 | 44.3 | 0.155 |
| DRB1*13:01 | 8 | 15.4 | 10 | 17.5 | 0.802 | 18 | 16.5 | 6 | 8.6 | 0.177 |
| DRB1*15:01 | 9 | 17.3 | 11 | 19.3 | 0.810 | 20 | 19.3 | 14 | 20 | 0.846 |
| DRB1*16:01 | 1 | 1.9 | 6 | 10.5 | 0.116 | 7 | 6.4 | 9 | 12.9 | 0.181 |
| DRB3 | 43 | 82.7 | 38 | 66.7 | 0.079 | 81 | 74.3 | 58 | 82.9 | 0.202 |
| DRB4 | 23 | 44.2 | 26 | 45.6 | 1.000 | 49 | 45.0 | 24 | 34.3 | 0.165 |
| DRB5 | 10 | 19.2 | 16 | 28.1 | 0.369 | 26 | 23.9 | 21 | 30 | 0.388 |

Less frequent alleles

| | Severe patients | | Moderate patients | | | Total patients | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Allele | N | F% | N | F% | P | N | F% | N | F% | P |
| A*23:01 | 1 | 1.9 | 1 | 1.8 | 1.000 | 2 | 1.8 | 3 | 4.3 | 0.381 |
| A*29:01 | 2 | 3.8 | 3 | 5.3 | 1.000 | 5 | 4.6 | 3 | 4.3 | 1.000 |
| B*07:05 | 3 | 5.8 | 1 | 1.8 | 0.346 | 4 | 3.7 | 0 | 0.0 | 0.157 |
| B*13:01 | 1 | 1.9 | 2 | 3.5 | 1.000 | 3 | 2.8 | 6 | 8.6 | 0.157 |
| B*15:03 | 1 | 1.9 | 4 | 7 | 0.366 | 5 | 4.6 | 0 | 0.0 | 0.158 |
| B*27:02 | 1 | 1.9 | 0 | 0.0 | 0.477 | 1 | 0.9 | 3 | 4.3 | 0.301 |
| B*39:01 | 1 | 1.9 | 2 | 3.5 | 1.000 | 3 | 2.8 | 3 | 4.3 | 0.680 |
| B*40:06 | 1 | 1.9 | 1 | 1.8 | 1.000 | 2 | 1.8 | 3 | 4.3 | 0.381 |
| B*44:02 | 0 | 0.0 | 5 | 8.8 | 0.058 | 5 | 4.6 | 0 | 0.0 | 0.158 |
| B*55:01 | 2 | 3.8 | 1 | 1.8 | 0.605 | 3 | 2.8 | 4 | 5.7 | 0.434 |
| B*57:01 | 4 | 7.7 | 0 | 0.0 | 0.059 | 4 | 3.7 | 1 | 1.4 | 0.650 |
| B*58:01 | 4 | 7.7 | 1 | 1.8 | 0.190 | 5 | 4.6 | 4 | 5.7 | 0.738 |
| DRB1*03:02 | 1 | 1.9 | 3 | 5.3 | 0.620 | 4 | 3.7 | 0 | 0.0 | 0.157 |
| DRB1*11:05 | 0 | 0.0 | 0 | 0.0 | – | 0 | 0.0 | 6 | 8.6 | 0.003** |
| DRB1*10:01 | 3 | 5.8 | 1 | 1.8 | 0.346 | 4 | 3.7 | 3 | 4.3 | 1.000 |
| DRB1*13:02 | 4 | 7.7 | 0 | 0.0 | 0.061 | 4 | 3.7 | 0 | 0.0 | 0.157 |
| DRB1*13:03 | 0 | 0.0 | 4 | 7 | 0.245 | 3 | 2.8 | 0 | 0.0 | 0.282 |
| DRB1*13:05 | 0 | 0.0 | 0 | 0.0 | – | 0 | 0.0 | 4 | 5.7 | 0.022* |
| DRB1*14:01 | 0 | 0.0 | 1 | 1.8 | 1.000 | 1 | 0.9 | 7 | 10 | 0.006* |

Abbreviations. F%: Allele frequencies (in percentage). N: Number of alleles. P: Value of Fisher's Exact Test analysis.

### 2.4. Statistical analysis

Statistical significance of differences in the frequencies of HLA-A, HLA-B, and HLA-DRB1 alleles between patient/control and moderate/severe patients was examined using Fisher´s Exact test. All statistical analyses were performed in SPSS 23. P-values $<0.05$ were considered statistically significant.

## 3. Results

### 3.1. Demographic and paraclinical characteristics of the participants

According to the statistical outcomes, the severe and moderate patients were homogenous in terms of gender and age (p: 0.86 and 0.26, respectively). The patient group was consisting of 52.3% moderate and 47.7% severe cases which are shown in detail in Table 2. Moreover, the patients were subdivided into 3 groups in terms of age including <40 years (ranging from 22 to 39), between 40 and 60 years, and >60 years old (ranging from 61 to 75). Clinical symptoms and laboratory data related to SARS-CoV-2 pathogenesis were recorded at the sampling time. Compared to moderate cases, the mean values of erythrocyte sedimentation rate, respiratory rate, AST, BUN, LDH, D-dimer, and ALT were significantly higher in severe patients, while the $O_2$ saturation rate was lower. Demographic and paraclinical data of the participants are provided in Table 2.

### 3.2. Association of HLA alleles and SARS-CoV-2 susceptibility and severity

Full analysis results of genotyped HLA alleles are presented in Table 3. Based on the statistical values, the frequencies of HLA-B*07:35 and HLA-DRB1*07:01 were associated with higher disease susceptibility as they were reported significantly more in patients rather than controls (p: 0.031 and 0.003 respectively). Moreover, HLA-DRB1*11:01 was observed more in severe patients as compared to moderates with a high significant value (p:<0.001). Also, HLA-DRB1*04:03 was reported more frequently in patients rather than control and in severe patients compared to moderate patients (p: 0.044 and 0.004, respectively). In contrast, because of higher frequencies in control cases, HLA-B*51:01, HLA-DRB1*11:05, HLA-DRB1*14:01, and HLA-DRB1*13:05 were reported to be involved in resistance to SARS-CoV-2 infection (p: 0.027, 0.003, 0.006 and 0.022, respectively). The statistical values indicated that the frequency of HLA-DRB1*04:01 was significantly higher in healthy subjects than patients and also in moderate patients rather than severe ones (p: 0.002).

### 3.3. Comparison of HLA alleles frequencies between deceased and recovered patients

Death is defined as a final clinical outcome of an infection or disease. To state a concise conclusion regarding the role of HLA alleles in COVID-19 mortality, the analysis of comorbidities, clinical data, and treatments should be considered. However, the main objective of this study was only to make a basic genetical evaluation in order to determine the presence or absence of the evaluated alleles. Nonetheless, using the Fisher's Exact Test, a primary comparison of HLA allele frequencies between deceased and discharged patients was done. The statistical values demonstrated that the frequencies of HLA-A*03, HLA-DRB1*11:01, and HLA-B*35 was significantly higher in deceased patients in comparison with discharged cases (p: 0.023, 0.018, and 0.043, respectively). Among the mentioned alleles, HLA-DRB1*11:01 was also reported higher in severe patients rather than moderates (p: <0.001). Therefore, this allele might play a role in disease severity and death caused by SARS-CoV-2 which is needed to be more clarified by larger investigations and multivariate analyses. Table 4 presents the statistical comparisons of the significant reported alleles.

## 4. Discussion

To fully understand the pathogenesis, prevention, and treatment of COVID-19, comprehensive clarifications on relation between genetical characterization of the host and SARS-CoV-2 are needed. It is undeniable that geographical differences may be responsible for the inconsistent findings in different populations leading to the variation of immune responses severity to viral infections [23,24]. Therefore, regional variations may significantly affect the outcomes and results of various studies. In this study, we performed a

**Table 4**
A comparison of HLA allele frequencies between deceased and discharged patients. This comparison was intended to provide a preliminary assessment of the difference in the frequencies of some alleles between deceased cases and recovered individuals and does not necessarily express the definite effect of these alleles in increasing mortality caused by COVID-19.

| Allele | | Death (N = 21) | Discharge (N = 88) | OR | P |
|---|---|---|---|---|---|
| HLA-A*03 | Yes | 8 (38.1%) | 12 (13.6%) | 3.897 | **0.023*** |
| | No | 13 (61.9%) | 76 (86.4%) | | |
| HLA-B*35 | Yes | 11 (52.4%) | 25 (28.4%) | 2.772 | **0.043*** |
| | No | 10 (47.6%) | 63 (71.6%) | | |
| HLA-DRB1*11:01 | Yes | 12 (57.1%) | 24 (27.3%) | 3.556 | **0.018*** |
| | No | 9 (42.9%) | 64 (72.7%) | | |

Abbreviations. N: Number of alleles OR: Odds ratio P: Value of Fisher's Exact test analysis. *p < 0.05.

molecular analysis in an Iranian population of the patients in order to screen the probable differences in the frequencies of HLA alleles between moderate and severe COVID-19 cases. It has been shown that HLA class I and class II genotypes may be involved in disease severity and clinical outcomes in COVID-19 patients. HLA class I plays a key role in directing effective immune response during viral infections such as SARS-CoV-2 [25]. The most polymorphic genes of HLA class I include HLA-B, HLA-C, and HLA-A which are more investigated in HLA-associated studies regarding COVID-19. Some of the previous researches are reported the probable association of the HLA-B*07:03, HLA-B*46:01, HLA-DRB1*03:01, and HLA-DRB1*12:02 alleles with the severity of SARS-CoV-2 due to their higher frequencies in COVID-19 patients with severe ARDS [26–28]. Moreover, Yung et al. have identified that HLA-B*22 serves as a potential risk factor for SARS-CoV-2 infection, and it is associated with susceptibility to SARS-CoV-2 in Chinese patients [29]. On the other hand, some studies have reported that a number of alleles are significantly more frequent in dead patients in comparison with severe and moderate ones. For instance, the results of a study in South Asia revealed that HLA-B*51 may be possibly associated with increased mortality risk in severe COVID-19 patients [30]. Additionally, HLA-B*35 frequencies was reported higher in mild group patients rather than fatal group [30]. M Shkurnikov et al. reported that the presence of the HLA-A*01:01 allele was associated with a high risk for COVID-19 mortality, while HLA-A*02:01 and HLA-A*03:01 mainly associated with a low risk of death in a group of the Russian population [31]. Also, a risk score (RS) analysis showed that HLA-A*01:01 homozygosity was accompanied by earlier deaths, however, only one HLA-A*02:01 homozygote died before 60 years of age [31]. A very important point that should be noted is that death is a clinical outcome of COVID-19 disease, and correlating it with some alleles simply by counting their frequencies in the group of deceased patients cannot indicate the certain association of these alleles with the increased risk of mortality. Therefore, death as a final outcome of the infection would need to be reconciliated with treatments, comorbidity and clinical data to assess the potential role of HLA alleles in disease mortality. It seems that high-resolution sequencing typing for HLA genes is associated with more precise results. A comparison between 137 mild and 70 severe patients with COVID-19 in the Center Hospital of the National Center for Global Health and Medicine (Tokyo, Japan) showed that the frequencies of HLA-A*11:01:01:01, HLA-C*12:02:02:01, and HLA-B*52:01:01:02 genotypes were higher in severe patients rather than mild patients [32]. In addition, multivariate analyses confirmed the significance of HLA-A*11:01:01:01, age at diagnosis, and sex as predictor factors involved in SARS-CoV-2 severity [32]. In terms of HLA class II, HLA-DRB1*09:01 was reported to be a significant factor contributing to severe COVID-19 development in Japanese patients [33]. Another analysis in Italian patients showed that HLA-DRB1*08 was associated with an increased risk of susceptibility and mortality [34]. In contrast, HLA-DRB1*01 was reported as a protective HLA genotype negatively correlated with the fatality rate of hospitalized COVID-19 patients in a large study in Mexico [35]. Our observations showed that HLA-DRB1*11:01 had a significantly higher frequency in severe and even dead patients. Conversely, in healthy individuals and moderately affected patients, the frequencies of HLA-DRB1*04:01 was significantly higher. The present study also revealed that HLA-DRB1*07:35 and HLA-DRB1*07:01 may be associated with increased susceptibility to SARS-CoV-2. However, due to the limitations including sample size, geographical variations, non-participation of ICU-admitted cases, different HLA typing methods, and highly variable clinical phenotype of SARS-CoV-2 infection, there are controversial reports on the involvement of HLA alleles in susceptibility to COVID-19 [36]. Ellinghaus et al. has conducted a genome-wide association study involving 1980 patients with severe disease at seven hospitals in Italian and Spanish SARS-CoV-2 epicenters. They analyzed 8,582,968 single-nucleotide polymorphisms by conducting a meta-analysis. They reported that there was no association between HLA alleles and COVID-19 infection [16]. Also, other similar studies in patients in Spain and Israeli, demonstrated the same results [37,38]. In contrast, Novelli et al. has analyzed the frequency distribution of HLA alleles in 99 severe or extremely severe COVID-19 patients from Italy, indicating a significant association between HLA-DRB1*15:01, HLA-DQB1*06:02, and HLA-B*27:07 [39]. Moreover, Kachuri et al. have conducted a genome-wide and transcriptome-wide association analysis to identify the association of HLA loci for 16 viruses in 7924 European participants. They also reported the probable importance of the HLA system in the host's response to viral infection [40]. Another study that was conducted by Wang et al. reported that HLA-C*07:29, HLA-C*08:01, HLA-B*15:27, HLA-B*40:06, HLA-DRB1*04:06, and HLA-DPB1*36:01 had higher frequencies, while HLA-DRB1*12:02 and HLA-DPB1*04:01 alleles had lower frequencies in COVID-19 patients. However, after the correction of the P-value, only HLA-C*07:29 and HLA-B*15:27 still remained significant [41]. One of the important limitations is the sample size that might affect the strength of the statistical analysis, especially for systems such as HLA with multiple alleles [39]. Although further multivariate studies are necessary to make a concise conclusion, our findings suggest that the mortality rate in COVID-19 patients may be associated with the presence of HLA-A*03, HLA-DRB1*11:01, and HLA-B*35 (Table 4). Toyoshima et al. has investigated 12,343 SARS-CoV-2 genome sequences of COVID-19 patients in six different geographic regions, revealing that the frequencies of several HLA alleles (e.g., HLA-A*11:01), as a dependent factor, was significantly associated with the virus fatality rate [42]. Overall, in the case of viral infection, HLA-DR serves as a double-edged sword. Ahout et al. have reported that phenotyping the monocyte subpopulations reveals a lower expression of HLA-DR during severe RSV infections. They explain how a reduced HLA-DR expression level indicates significant differences between severe and non-severe RSV infections as well as an imbalanced innate immune response, which may lead to the higher severity of the disease [43]. In contrast, Spriggs et al. have reported that HLA-DR serves as a co-factor for the infection of B lymphocytes by the Epstein-Barr virus. It is well documented that three glycoproteins gp42, gL, and gH are involved in of EBV attachment and internalization to the cell membrane. Among these glycoproteins, gp42 binds to HLA-DR and interferes with this interaction via a monoclonal antibody inhibit the infection [44]. SARS-CoV-2 genome variants may also affect peptide binding affinity to the HLA molecules. Sousa et al. have confirmed that a wild-type sequence of SARS-CoV-2 affected by a single mutation can influence the peptide binding of virus variants only to the MHC class II (e.g., HLA-DR) and plays a key role in the clinical outcome and disease severity of COVID-19 [45]. Our results suggest that HLA-DRB1*11:01 is associated with the severity of COVID-19 among patients in Iran. In contrast, the presence of HLA-DRB1*04:01, HLA-B*51:01, HLA-DRB1*11:05, HLA-DRB1*13:05, and HLA-DRB1*14:01 may indicate disease resistance. Overall, the finding of HLA association with COVID-19 severity or resistance is closely dependent on the targeted population and regional variations as described by previous comprehensive investigations in different populations [36,38]. It

seems that the evaluation of peptide-binding grooves of those HLA alleles may help describe their association with COVID-19 severity. Based on the results published in different populations, it seems that regional differences are one of the most important factors that determine the severity of SARS-CoV-2 infection. Certainly, conducting these studies on a larger scale with a larger number of samples can provide more reliable results, but the importance of the alleles that have been reported so far should not be neglected. It is very important to conduct functional studies for bioinformatics and in vitro evaluation of the effect of identified alleles on the activation of antiviral lymphocyte clones. The use of alleles related to reducing the spread and damage of the virus in order to design an effective vaccine and treatment method must be considered.

## 5. Conclusions

HLA system plays a critical role in managing the immune system response during viral infections. Based on the findings in the literature, there are inconsistent results in different populations and geographical areas on the role of HLA allele frequencies in COVID-19 severity. On the other hand, highly variable clinical phenotype of the patients is probably impacting the results. Therefore, according to the limitations of such studies as well as the diversity in the obtained results, it seems that in each population, different alleles play a role in the exacerbation and occurrence of the disease. As a result, it can be stated that the significant alleles reported in this article can be considered as research targets to be more investigated in Iranian COVID-19 patients. Conducting multivariate statistical assessments on the relation between reported alleles and clinical parameters of the disease severity would be helpful to clarify the exact role of mentioned HLA alleles in disease progression.

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## Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the institution's policy to code and archive data in a central repository of the hospital, but are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences has approved the study (IR.AJUMS.REC.1399.142). All the participants in the study were informed and agreed to be included in the project. Informed consents were obtained from all the patients.

## CRediT authorship contribution statement

**Farhad Abolnezhadian:** Conceptualization, Funding acquisition, Validation. **Sara Iranparast:** Supervision, Visualization. **Mojtaba Shohan:** Investigation, Validation, Writing – review & editing. **Zahra Shokati Eshkiki:** Writing – original draft, Investigation, Software. **Mahtab Hamed:** Resources, Formal analysis, Methodology. **Maryam Seyedtabib:** Formal analysis, Resources, Methodology. **Roohangiz Nashibi:** Project administration, Conceptualization, Supervision. **Mohammad-Ali Assarehzadegan:** Formal analysis, Investigation. **Seyed Ali Mard:** Investigation, Software, Writing – original draft. **Ali Akbar Shayesteh:** Conceptualization, Project administration. **Niloofar Neisi:** Investigation, Formal analysis. **Manoochehr Makvandi:** Investigation, Formal analysis. **Seyed Mohammad Alavi:** Conceptualization, Project administration, Supervision. **Gholamreza Shariati:** Conceptualization.

## Declaration of competing interest

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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## Abbreviations

**AST** Aspartate aminotransferase
**ALT** Alanine transaminase
**ALP** Alkaline phosphatase
**Alb** Albumin

**BUN** Blood urea nitrogen
**Cr** Creatinine
**CTL:** Cytotoxic T lymphocytes
**EBV** Epstein Barr virus
**ESR** Erythrocyte sedimentation rate
**HLA** Human leucocyte antigens
**IDSA** Infectious Diseases Society of America
**MHC** Major histocompatibility complex
**PR** Pulse rate
**RR** Respiratory rate
**RSV** Respiratory syncytial virus
**SARS-CoV-2** Severe acute respiratory syndrome coronavirus 2
**SSP-PCR** Sequence specific primer polymerase chain reaction

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