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Original Article

Study of *HLA-A*, *-B*, *-C*, *-DRB1* and *-DQB1* polymorphisms in COVID-19 patients

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Abstract *Background:* Human leukocyte antigen (HLA) plays an important role in immune responses to infections, especially in the development of acquired immunity. Given the high degree of polymorphisms that HLA molecules present, some will be more or less effective in controlling SARS-CoV-2 infection. We wanted to analyze whether certain polymorphisms may be involved in the protection or susceptibility to COVID-19.
Methods: We studied the polymorphisms in HLA class I (HLA-A, -B and -C) and II (HLA-DRB1 and HLA-DQB1) molecules in 450 patients who required hospitalization for COVID-19, creating one of the largest HLA-typed patient cohort to date.
Results: Our results show that there is no relationship between HLA polymorphisms or haplotypes and susceptibility or protection to COVID-19.

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Conclusion: Our results may contribute to resolve the contradictory data on the role of HLA polymorphisms in COVID-19 infection.

Introduction

COVID-19 (Coronavirus Disease 2019) is a respiratory tract infection, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that can progress to pneumonia, acute respiratory distress syndrome (ARDS), cytokine storm, multiple organ failure, and death.[1] The severity of COVID-19 depends on increased age, and comorbidities such as obesity, arterial hypertension, diabetes, heart disease and respiratory disease.[2,3] There is a great need to study factors that play a relevant role in host defense, with the objective of identifying variables of susceptibility and severity. Of special interest are the human leukocyte antigens (HLA), which are highly polymorphic proteins that play a crucial role in the function of adaptive immunity. HLA presents pathogen-derived peptides on the surface of the infected cell, which are then recognized by specific T lymphocytes, inducing an immune response against the pathogen.[4–7] The high level of polymorphism found in both HLA class I (HLA-A, -B and –C) and class II (HLA-DP, -DQ, -DR) molecules increases the variety of peptides that can be presented and recognized by the immune system. This is due to variations in their physical/chemical properties that increases or decreases their ability to bind and present certain peptides. Because of this, an allele can be a good or poor presenter of peptides derived from a pathogen. There is evidence of HLA alleles that can play a protective or susceptible role in infections caused by human immunodeficiency virus (HIV), hepatitis C virus (HCV), influenza virus and plasmodium.[8,9]

Each person has a different combination of HLA alleles (haplotypes) that determines their ability to respond to certain pathogens. These haplotypes have been selected throughout evolution due to the selective pressure carried out by pathogens.[10] It has been suggested that the differences observed in the number of cases and severity of COVID-19 between different regions of the world, may in part be due to a skewed distribution of HLA alleles involved in protective immunity against SARS-CoV-2.[11,12]

Interestingly, there are studies that link HLA alleles to SARS-CoV-1, a coronavirus closely related to SARS-CoV-2. The HLA-B*46:01 allele has been linked to disease severity in SARS-CoV-1 patients, while the HLA-B*07:03 and HLA-DRB1*03:01 alleles are related to susceptibility to SARS-CoV-1.[13,14]

Therefore, we hypothesize that HLA typing of patients infected by SARS-CoV-2 can help us find alleles that are involved in susceptibility, protection, and poor prognosis to COVID-19. To this end, we have performed a high-resolution HLA typing of 450 confirmed SARS-CoV-2 patients who required hospitalization, comparing their allele and haplotype frequencies with a group of 959 representative controls. We believe that such an analysis can generate data that could greatly aid the development of personalized treatments, diagnosis and vaccination.[15]

Materials and methods

Patients

The current study was performed on 450 hospitalized COVID-19 patients at the Hospital Universitario Virgen de las Nieves, Granada, Spain. The samples were collected from April 2020 to January 2021. The inclusion criteria were the need for hospitalization due to pneumonia or respiratory distress due to COVID-19, diagnosed by a positive SARS-CoV-2 PCR or the presence of SARS-CoV-2-specific IgG antibodies in blood as described below. The characteristics and comorbidities of the patient group can be seen in Table 1.

Table 1 Description of the hospitalized COVID-19 patients and comorbidities.

Features	Mean (range) or n (%)
Age (years)	62 (25—98)
Female	220 (45.5%)
Male	263 (54.5%)
Diagnosis by PCR	363 (75.2%)
Diagnosis by antibodies	120 (24.8%)
ICU	126 (26.1%)
No ICU	324 (73.9%)
Mechanic Ventilation	101 (20.9%)
No Mechanic Ventilation	349 (79.1%)
Deceased	70 (14.5%)
Survivors	380 (85.5%)
Comorbidities	**n (%)**
Hypertension	196 (40.6%)
DM	101 (20.9%)
Overweight/Obesity	72 (14.9%)
CKD	33 (6.8%)
COPD	32 (6.6%)
Asthma	26 (6%)
MI	26 (5.4%)
HF	25 (5.2%)
CVD	22 (4.6%)
PAD	11 (2.3%)

n: patient number; ICU: Intensive Care Unit; DM: Diabetes Mellitus; CKD: Chronic Kidney Disease; CVD: Cerebrovascular Disease MI: Myocardial infarction; HF: Heart Failure; COPD: Chronic obstructive pulmonary disease; PAD: Peripheral Artery Disease.

The patients had pneumonia or respiratory distress and were classified according to severity of the disease in need to enter intensive care unit (ICU)/No ICU; need for Mechanical Ventilation/No Mechanical Ventilation; Deceased/Survivors (Table 1).

The control group (n = 959) is made up of healthy blood donors, representative of the Granada area, who were not infected at the time of donation. The average age of the group is 45 ± 5 years and 51% of the members are women.

The study was reviewed and approved by the Portal de Ética de la Investigación Biomédica. Junta de Andalucía (Cod. 0766-N-20). The patients/participants provided their written informed consent to participate in this study.

PCR and serological diagnosis of SARS-CoV-2 infection

For PCR diagnosis we use the cobas® SARS-CoV-2 assay on a cobas® 6800 system (Roche Molecular Systems, Pleasanton, California, USA). This is a single-well, double-target assay that enables both the specific detection of SARS-CoV-2 and the detection of pan-Sarbecovirus of the Sarbecovirus subgenus family, which includes SARS-CoV-2. The test detects the genetic signature (RNA) of the SARS-CoV-2 virus in nasal, nasopharyngeal and oropharyngeal swab samples from patients who meet COVID-19 clinical and/or epidemiological criteria for testing.

The ARCHITECT i System platform (Abbott Laboratories, Chicago, Illinois, USA), was used with the SARS-CoV-2 IgG reagent (Abbott Laboratories, REF. 6R86-22), which is a chemiluminescent microparticle immunoassay (CMIA), used for the qualitative detection of IgG antibodies against the SARS-CoV-2 virus. The results were considered positive when a value ≥ 1.4 URL was obtained and negative when it was <1.4 URL.

DNA extraction and determination of HLA class I and II genotypes

Venous blood was obtained from each patient and DNA was extracted using the QIAMP DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. High-resolution genotyping of HLA class I (A, B and C) and II (DRB1, DQB1 and DPB1) loci was performed using the LABType sequence-specific oligonucleotide typing test (One Lambda, Canoga Park, California, USA). Target DNA was amplified by PCR using sequence-specific primers, followed by hybridization with allele-specific oligodeoxynucleotides coupled with fluorescent phycoerythrin-labelled microspheres. Fluorescence intensity was determined using a LABScan 100 system (Luminex xMAP, Austin, Texas, USA). HLA alleles were assigned using the HLA-Fusion software (One Lambda).

Statistical analysis

Statistical tests for alleles, genotypes and haplotypes were performed using the R (R software; R Foundation for Statistical Computing) package BIGDAWG.[16] Hardy—Weinberg equilibrium (HWE) tests were calculated using PyPop software ver. 0.7.0 (http://www.pypop.org). Frequencies of individual HLA alleles in patients and controls were compared using the $\chi 2$-test. Variants with expected counts less than five were combined into a common class (binned) prior to computing the $\chi 2$-test.

The R package BIGDAWG was also used for amino acid analysis. The software uses the collection of alleles in the input dataset, to retrieve a list of aligned amino acid sequences from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/align.html) and to run case—control association tests on individual amino acid positions within exon 2 and exon 3 (HLA class I) or exon 2 (HLA class II).

Significance levels were corrected by Bonferroni correction for multiplicity of testing by the number of comparisons. A corrected P value of <0.05 was considered statistically significant for all statistical tests.

Results

HLA-allele and haplotype analysis

Genotype frequencies of the HLA-A, -B, —C, -DRB1 and -DQB1 loci did not deviate from Hardy-Weinberg expectations (Supplementary Table 1).

The comparison of the HLA allele frequencies between the control group and hospitalized COVID-19 patients identified several alleles with significantly different frequencies. However, the significances were lost after Bonferroni correction (Supplementary Table 2).

The HLA-A*25:01 and DRB1*11:01 alleles had a significantly higher frequency in the control group without correction (Table 2). These alleles could represent possible protective alleles to COVID-19.

The alleles HLA-A*66:01, B*40:02, B*55:01, DRB1*14:04 and DQB1*05:03 also presented significant values without correction, being overrepresented in hospitalized COVID-19 patients (Table 2). These alleles could be considered as susceptibility alleles to COVID-19.

The haplotype study did not show significant results either, but the extended haplotype HLA-A*29:02, B*44:03, C*16:01, DRB1*07:01 and DQB1*02:02 is of interest, which without correction had a p-value of 0.013 (Table 3).

To address the relationship between HLA and disease severity, we compared the allelic and haplotypic frequencies between the following groups: patients admitted to ICU versus patients that no admitted to ICU; patients that needed mechanical ventilation versus patients that did

Table 2 Alleles with the P values closest to significance.

Alleles	Control group frequencies	Hospitalized COVID-19 frequencies	P	Pc
HLA-A*25:01	**0.0175**	0.0044	0.005	n.s
HLA-DRB1*11:01	**0.0695**	0.0464	0.017	n.s
HLA-A*66:01	0.0058	**0.0133**	0.042	n.s
HLA-B*40:02	0.0096	**0.0199**	0.024	n.s
HLA-B*55:01	0.0074	**0.0166**	0.026	n.s
HLA-DRB1*14:04	0.0032	**0.0121**	0.005	n.s
HLA-DQB1*05:03	0.0287	**0.0453**	0.024	n.s

The highest frequency for each allele is marked in bold. Pc: P corrected by Bonferroni. n.s: not significant.

not require mechanical ventilation; deceased patients versus surviving patients; and between the aforementioned groups and controls. These comparisons did not result in any significant differences after Bonferroni correction (data not shown).

HLA supertype and amino acid analysis

Most of the polymorphism in HLA molecules is located in the peptide-binding region. Despite being extremely polymorphic, HLA-A and HLA-B alleles can be clustered into supertypes, representing families of molecules that share an overlapping peptide binding specificity.[17]

To investigate the clinical relevance of peptide binding specificity, we examined the effects of HLA-A and HLA-B supertypes on COVID-19 risk (Table 4). The comparison of the HLA-A and HLA-B allele supertype frequencies between the control group and COVID-19 patients did not show any significant differences.

In a further step, we performed an amino acid analysis of the HLA alleles identified in patients and controls. We examined individual amino acid positions within exon 2 and exon 3 (class I) or exon 2 (class II). These exons include the peptide binding groove and are the most polymorphic. After Bonferroni correction no specific amino acids in the HLA loci A, B, C, DRB1 or DQB1 were found to provide any significant contribution to COVID-19 risk or protection (Supplementary Table 3).

The amino acid analysis was also performed between the different severity groups: patients admitted to ICU versus patients that no admitted to ICU; patients that needed mechanical ventilation versus patients that did not require mechanical ventilation; deceased patients versus surviving patients; and between the aforementioned groups and controls, not finding significant values after Bonferroni correction (data not shown).

Table 4 HLA supertypes in COVID-19 patients and controls.

	Controls	Patients	OR	P
HLA A-supertypes				
A01	22.65	23.03	1.03	0.7872
A01 A03	2.07	1.36	0.66	0.2068
A01 A24	8.98	8.86	0.99	0.9326
A02	27.01	27.52	1.03	0.7362
A24	13.53	13.22	0.98	0.8428
A03	25.43	25.48	1.01	0.9392
Unclassified	0.12	0.00	0	0.3591
HLA B-supertypes				
B27	14.47	15.26	1.06	0.6056
B44	34.91	34.06	0.96	0.5992
B58	4.92	4.50	0.91	0.6086
B62	5.24	4.36	0.82	0.3071
B07	28.80	31.34	1.12	0.1836
B08	5.05	5.04	0.99	0.9696
Unclassified	5.65	4.91	0.86	0.4003

OR: odds ratio; P: P-value.

Discussion

Our results suggests that there is no significant correlation between particular HLA alleles/haplotypes and susceptibility or protection against COVID-19, which is in agreement with a previous study.[18] However, numerous studies have reported alleles of protection or susceptibility to COVID-19. A study conducted with 82 Chinese patients found that the HLA-C∗07:29 and HLA-B∗15:27 alleles were more frequently detected in the COVID-19 group than in the control population.[19] Novelli et al., found that the HLA-B∗27:07, DQB1∗06:02 and -DRB1∗15:01 alleles were significantly increased in a group of 99 COVID-19 Italian patients compared to the control group.[20] In addition, the alleles HLA-B∗44 and C∗01 were positively and individually associated with COVID-19 in the Italian population.[21] The HLA-A∗02:01 allele has a possible positive association with the risk of COVID-19.[22] Another study, with 190 Chinese patients, found a positive correlation between the HLA-B22 serotype and COVID-19 susceptibility.[23] These studies were carried out with a smaller number of patients compared to our study, which could be one of the reasons as to why we could not detect the above associations. Furthermore, Correale et al.[21] and Tomita et al.,[22] did not

Table 3 Frequencies of most represented haplotypes in COVID-19 hospitalized patients and controls.

A ~ B ~ C ~ DRB1 ~ DQB1	Controls	Patients	OR	P	Pc
01:01~08:01~07:01~03:01~02:01	0.0180	0.0132	0.73	0.3510	
02:01~07:02~07:02~01:03~05:01	0.0042	0.0099	2.35	0.0707	
02:01~07:02~07:02~15:01~06:02	0.0149	0.0132	0.89	0.7365	
02:01~18:01~05:01~03:01~02:01	0.0111	0.0077	0.69	0.3960	
03:01~07:02~07:02~15:01~06:02	0.0106	0.0132	1.25	0.5414	
11:01~27:05~01:02~01:01~05:01	0.0064	0.0066	1.04	0.9377	
11:01~35:01~04:01~01:01~05:01	0.0069	0.0044	0.64	0.4296	
23:01~44:03~04:01~07:01~02:02	0.0069	0.0077	1.12	0.8086	
29:02~44:03~16:01~07:01~02:02	0.0281	0.0464	1.68	**0.0129**	n.s
30:02~18:01~05:01~03:01~02:01	0.0191	0.0254	1.34	0.2805	
33:01~14:02~08:02~01:02~05:01	0.0111	0.0077	0.69	0.3960	

P values written in bold are significant. Variants with expected counts less than five were not computed in the $\chi 2$-test. OR: odds ratio. Pc: P corrected by Bonferroni. n.s: not significant.

perform HLA typing, but instead used the allelic frequencies in certain defined geographical regions and the incidence of COVID-19 in these regions. Importantly, our work contains one of the largest HLA-typed COVID-19 patient cohort to date.

Studies of the relationship between HLA alleles and the development of COVID-19 have yielded both similar and contrasting results. The alleles HLA-A∗11:01, C∗04:01 and DQA1∗01:02 have been associated with a worse evolution of the disease.[24] Furthermore, the HLA-A∗11, C∗01, DQB1∗04[25,26], and DQB1∗08[27] alleles have all been associated with a higher mortality among COVID-19 patients. Interestingly, the HLA-C∗05 allele has been associated with risk of death,[28] while the work done by Poulton et al., speaks of a protective role of this allele.[29] It is interesting to note the discrepancies between different studies with regard to protection, risk or development of COVID-19, the ethnicity of the patients/control individuals may play a role.

In silico studies have identified alleles that are better or worse presenters of conserved SARS-CoV-2 peptides. The HLA-A∗02:02, B∗15:03 and C∗12:03 alleles are considered the best presenters of conserved peptides of SARS-CoV-2 while the HLA-A∗25:01, B∗46:01 and C∗01:02 alleles are poor presenters.[30] These data were confirmed by La Porta et al., who in addition identified HLA-A∗11:01 as a good presenter.[31] These results were corroborated by the study carried out by Barquera et al., which also showed that the HLA class II alleles DRB1∗01:01, DRB1∗10:01, DRB1∗11:02 and DRB1∗13:01 present more SARS-CoV-2 peptides whereas the HLA-DRB1∗03:02, DRB1∗03:03 and DQA1∗01:02/DQB1∗06 alleles were identified as the worst presenters of SARS-CoV-2-derived peptides.[32]

It is logical to think that patients requiring hospitalization have HLA alleles that do not bind strongly immunogenic SARS-CoV-2 peptides, resulting in an ineffective control of the disease by the immune system. This has been described by Iturrieta-Zuazo et al., who determined that patients with mild disease have HLA alleles with a greater theoretical binding capacity to SARS-CoV-2 peptides.[33]

In contrast, our data showed that the poor presenting HLA-A∗25:01 allele was underrepresented in hospitalized COVID-19 patients, compared to the control group (Table 2). We only found three patients with the HLA-A∗25:01 allele in our cohort of seriously ill patients who required hospitalization. These results are in agreement with the study by Wang et al., who reported that the HLA-B∗46:01 allele, the worst presenter of peptides derived from SARS-CoV-2, is increased in patients with milder disease compared to those with serious illness.[34] However, we were not able to analyze the HLA-B∗46:01 allele since it was absent in both the control and COVID-19 hospitalized group.

In summary, contrary to our expectations, we observed that critically ill patients had a lower frequency of HLA alleles with poor virus presenting capacity (HLA-A∗25:01). These results, together with those of Wang et al.,[34] lead us to think of a possible hypothesis, in which the alleles with the lowest ability to present peptides derived from SARS-CoV-2 may be related with a protective role.

The clinical manifestations in severe COVID-19 patients are characterized by unilateral or bilateral pneumonia, with a state of hyperinflammation due to the activation of the IL-1 or IL-6 pathways that can evolve into a cytokine storm.[35] An increase in pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α and IFN-γ),[36] as well as lymphopenia and an increase in the activation of the few remaining lymphocytes have been described in the most severe cases.[37] Likewise, the increased activation of CD8+ T lymphocytes,[38] possibly due to the high amount of cytokines, can cause great damage to the lung epithelium, resulting in to hypoxemia, hypotension and even shock.[35] We hypothesize that an efficient presentation of SARS-CoV-2-derived would increase the cytotoxic CD8+ T cell response, augmenting tissue damage in the lungs and elevating the risk of death in severe COVID-19 patients. In addition, it would produce an increase in the activation of T helper lymphocytes (Th), further promoting the inflammatory state. Patients with more severe disease have been shown to have broader and stronger T cell responses,[38] which may be due to a high viral load due to poor viral control by innate immunity,[38] an insufficient early T cell response or to a better recognition of the virus by lymphocytes.

However, our results suggest that there is no relationship between COVID-19 and HLA polymorphisms, also supported by supertypes and amino acid analysis, indicating an irrelevant role of HLA in the risk of COVID-19 infection, which is in agreement with recent genome-wide association studies (GWAS).[39] In fact, the GWAS identified a polymorphism in IFNAR2 (Interferon alpha and beta receptor subunit 2) related to COVID-19.[39] Given this, we believe that innate immunity is crucial for viral infection control, with interferon playing an important role.[40] The virus has numerous mechanisms to bypass the innate immune system and block an effective interferon response.[41] A poor response in the initial stages of infection, would lead to an increase in viral load and a poor prognosis of the disease.[42] The role of HLA in COVID-19 may be to generate robust T cell responses in cases where innate immunity has managed to efficiently control infection. However, in the most severe patients, where it is very possible that the viral load is high and a state of hyperinflammation is present, the HLA proteins that best present viral peptides could worsen the patient's condition by inducing a greater activation of Th and CD8 + cytotoxic lymphocytes, increasing the inflammatory state and tissue damage.

Finally, our data do not show significant values for haplotypes. In contrast, an Italian study found a positive significant correlation between the HLA-A∗01:01, B∗08:01, C∗07:01 and DRB1∗03:01 haplotype and the incidence and death from COVID-19 (haplotype of susceptibility).[43] In addition, the haplotype HLA-A∗02:01, B∗18:01, C∗07:01 and DRB1∗11:04 was inversely related to the incidence and death by COVID-19 (haplotype of protection).[43] Another study carried out in Sardinia identified a protective role of the haplotype HLA-A∗02:05, B∗58:01, C∗07:01, DRB1∗03:01.[44] Finally, the study carried out by Wang et al., showed that the HLA-A∗11:01, B∗51:01 and C∗14:02 haplotype was associated with more severe COVID-19.[34] The HLA-A∗29:02, B∗44:03, C∗16:01, DRB1∗07:01 and DQB1∗02:02 haplotype, which was close to significance in our study, presented almost a 2-fold higher frequency in the COVID-19 hospitalized group versus control group (Table 3). Interestingly, the frequency of this haplotype was significantly ($P = 0.006$ without correction) higher (more that 2-fold) in the ICU patients versus control group. These data may suggest that this haplotype is related to severity and admission to the ICU. The frequency

of this haplotype is 6.2% in the Spanish population,[45] and may be one of the explanations to the high incidence of severity and mortality that the country has suffered.

Based on the results of our study, without taking into account the possibility that certain HLA alleles might modify the clinical course of the disease, we can conclude that HLA polymorphism is not a determining factor in the risk of COVID-19 infection. Furthermore, the allelic-level comparisons made by Ellinghaus et al. with a Spanish and Italian population, further reinforce our results.[46] Moreover, our results agree with the study by Shachar et al., published during the revision of our manuscript, where they found no relationship between HLA polymorphisms and haplotypes in the Israeli population.[47] In addition, the impact of HLA polymorphisms on the outcome (risk/protection) of COVID-19 might by partially masked by the current improvements in patient care and the possible emergence of antivirals, and might be appreciated with greater clarity in the long term. Further studies are needed to answer these important questions.

Author contributions

F. R—C and M.A. L-N contributed to the design of the study. M. A. L-N, A. M-c and A. F-R performed the HLA typing. A. R—N performed the statistical analysis of the data. M. A. L-R and J. F. G-B collected samples from hospitalized COVID-19 patients. J. F. G-B, F. R—C, A. R—N and P. A wrote the manuscript. A. R—C and M. A. L-R contributed to the clinical follow-up of patients. All authors contributed to manuscript revision, read and approved the submitted version.

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Institutional review board statement

The studies involving human participants were reviewed and approved by Portal de Ética de la Investigación Biomédica. Junta de Andalucía (Cod. 0766-N-20). The patients/participants provided their written informed consent to participate in this study.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.08.009.