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Cross-reactive humoral immune responses against seasonal human coronaviruses in COVID-19 patients with different disease severities

Kazuo Imai, Masaru Matsuoka, Sakiko Tabata, Yutaro Kitagawa, Mayu Nagura-Ikeda, Katsumi Kubota, Ai Fukada, Tomohito Takada, Momoko Sato, Sakiko Noguchi, Shinichi Takeuchi, Noriaki Arakawa, Kazuyasu Miyoshi, Yoshiro Saito, Takuya Maeda

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Background: The cross-reactive antibody response against seasonal human coronaviruses (HCoVs) was evaluated according to disease severity in patients with COVID-19 in Japan.

Methods: In total, 194 paired serum samples collected from 97 patients with COVID-19 (mild, 35; severe, 62) were analyzed on admission and during convalescence. IgG antibodies against the nucleocapsid (N) and spike (S) proteins of SARS-CoV-2 and four seasonal HCoVs (HCoV-NL63, -229E, -OC43, and -HKU1) were detected by enzyme-linked immunosorbent assays.

Results: There was no difference in optical density (OD) values for seasonal HCoVs on admission between the severe and mild cases. In addition, a specific pattern of disease severity-associated OD values for HCoVs was not identified. Significant increases in OD values from admission to convalescence for HCoV-HKU1 and -OC43 IgG-S, and for HCoV-NL63 and -229E IgG-N were observed in the severe cases. Significant differences were observed between the mild and severe cases for HCoV-HKU1 and -OC43 IgG-S OD values during convalescence. Correlations were found between the fold changes for HCoV-OC43 IgG-S OD values, and for SARS-CoV-2 IgG-S OD values, and C-reactive protein, lactate dehydrogenase, and lymphocyte levels.

Conclusion: There was no association between the antibody titer for seasonal HCoVs in the early phase of COVID-19 and disease severity.

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Introduction

Novel coronavirus disease 2019 (COVID-19), which is caused by the lineage B betacoronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is an ongoing pandemic. An understanding of the humoral immune response against SARS-CoV-2 is important for assessing the potential for viral recombination, diagnosis, surveillance, and pathogenesis. SARS-CoV-2 contains four major structural proteins, including the spike (S), membrane (M), and envelope (E) proteins that form the viral envelope, and the nucleocapsid (N) protein, which is located in the ribonucleoprotein core. The S protein, which consists of two subunits (S1 and S2), is responsible for recognition of the host cellular receptor that initiates virus entry (Cui et al., 2019). In addition, SARS-CoV-2 encodes 16 non-structural proteins and nine accessory proteins (Dai and Gao, 2021). The S and N proteins are highly immunogenic for humoral responses, whereas the M and E proteins are poorly immunogenic (Du et al., 2008; Long et al., 2020). Therefore, S and N are considered the main antigens of SARS-CoV-2. Our understanding of the humoral immune responses of patients with...
COVID-19 continues to increase as a result of the ongoing efforts of the worldwide scientific community (Imai et al., 2021; Liu et al., 2020; Long et al., 2020; Ma et al., 2020; Zhao et al., 2020). Interestingly, several studies have reported the simultaneous seroconversion of IgM and IgG against the S and N antigens of SARS-CoV-2 in patients with COVID-19, as observed in the second infection pattern (Imai et al., 2021; Long et al., 2020). Thus, it has been hypothesized that patients with COVID-19 could develop cross-reactivity of the immune response between previous infections with other seasonal human coronaviruses (HCoVs) and SARS-CoV-2, with differences in antibody response and disease severity perhaps the result of previous infections with seasonal HCoVs.

Four seasonal HCoVs (HCoV-NL63, -229E, -OC43, and -HKU1) have been identified as human pathogens. Seasonal HCoVs cause respiratory tract infections in humans, are detected most frequently in winter, and are responsible for 15–30% of common colds worldwide (Liu et al., 2020b). Seasonal HCoVs belong to two distinct taxonomic genera: HCoV-NL63 and -229E belong to the alphacoronavirus family; HCoV-OC43 and -HKU1 belong to the betacoronavirus family (Liu et al., 2020b). Protein homology between SARS-CoV-2 and seasonal HCoV antigens is approximately 19.8% for S1, 39.9% for S2, and 33.0% for N (Saletti et al., 2020). Potential cross-reactivity of immunity between SARS-CoV-2 and seasonal HCoVs has been reported, but it is unknown whether the humoral immunity induced by previous infections with seasonal HCoVs can prevent disease exacerbation (Dugas et al., 2021; Greinbaum et al., 2021; Hicks et al., 2021; Ha et al., 2020; Mateus et al., 2020; Ng et al., 2020; Ringlander et al., 2021; Sermet-Gaudelus et al., 2021; Wang et al., 2020a). It is also unknown whether there is an association between the antibody cross-reactivity patterns of seasonal HCoVs and the severity of COVID-19.

In our study, the cross-reactivity of humoral immunity for the S and N proteins of seasonal HCoVs and the severity of COVID-19 was evaluated using 194 paired serum samples collected during the early infection and convalescence periods from cases with mild and severe COVID-19.

Methods

Ethical statement

This study was reviewed and approved by the Institutional Review Boards of the National Institute of Health Sciences (approval number 333), Saitama Medical University Hospital (approval number 20123.01), and Self-Defense Forces Central Hospital (approval number 02-046).

Patients with COVID-19, clinical data, and serum samples

Patient information was collected retrospectively from the hospital medical records, and included clinical records and laboratory findings. In total, 97 patients with COVID-19 who were hospitalized at Saitama Medical University Hospital and the Self-Defense Forces Central Hospital in Japan from February 11 to November 23, 2020, were enrolled. SARS-CoV-2 RNA was detected in all patients with COVID-19 by quantitative reverse-transcription polymerase chain reaction (RT-qPCR) (Shirato et al., 2020) and reverse-transcription loop-mediated isothermal amplification (RT-LAMP; Loopamp® 2019-SARS-CoV-2 Detection Reagent Kit; Eiken Chemical, Tokyo, Japan), according to the Infectious Disease Law in Japan. Patient age ranged from 18 to 94 years (median, 61 years; interquartile range [IQR], 48–74 years); 72 patients (74.2%) were male and 25 (25.8%) were female. According to their presentation during hospitalization, the symptomatic cases were subdivided retrospectively into two groups (mild and severe) at the end of hospitalization to facilitate statistical analysis based on the number of cases. The classification of severity was adapted from the Guidelines on the Diagnosis and Treatment of Novel Coronavirus issued by the Ministry of Health, Labour, and Welfare, Japan. Severe symptomatic cases were defined as patients showing clinical symptoms of pneumonia (percutaneous oxygen saturation < 93%) and in need of oxygen therapy. The remaining symptomatic patients were classified as mild cases. Finally, among the 97 patients, 35 (36.1%) and 62 (63.9%) were classified as having mild and severe COVID-19, respectively. Nasopharyngeal specimens homogenized in phosphate-buffered saline (PBS) were collected on admission and stored at −80°C until RT-qPCR analysis for seasonal HCoVs. The 194 paired serum samples were collected from each patient on admission and during convalescence (at ≥ 18 days after initial symptom onset). The median period from initial symptom onset to admission was 8 days (IQR, 7–13 days), and to convalescence, 20 days (IQR, 18–24 days). All serum samples were stored at −80°C.

Molecular diagnostic methods for SARS-CoV-2

For the initial diagnosis of COVID-19, RT-qPCR and RT-LAMP were performed according to the National Institute of Infectious Diseases (NIID) protocol, which is recommended for SARS-CoV-2 detection in Japan (Shirato et al., 2020). A nasopharyngeal swab collected from each patient was homogenized in 1.0 mL PBS, and viral RNA was extracted from 140 μL of this solution using a QIAasymp©® RNA Kit (Qiagen, Hilden, Germany) and a High Pure Viral RNA Kit (Roche, Basel, Switzerland), according to the manufacturers’ instructions. The gene encoding the N protein of SARS-CoV-2 was amplified by RT-qPCR using a Quantitect Probe RT-qPCR Kit (QiAGEN) and the following primers and probe: NIID_2019-nCOV_N_F2, NIID_2019-nCOV_N_R2, and NIID_2019-nCOV_N_P2 (Shirato et al., 2020). Amplification was performed under the following conditions: reverse transcription at 50°C for 30 min; initial denaturation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 15 s; and annealing/extension at 60°C for 60 s. A positive result, indicating the presence of SARS-CoV-2 RNA, was determined according to the Ct value: Ct values < 40 indicated the presence of the virus. RT-LAMP detection of SARS-CoV-2 RNA was performed using a Loopamp® 2019-SARS-CoV-2 Detection Reagent Kit (Eiken Chemical) following the manufacturer’s instructions. A nasopharyngeal swab was homogenized in 4 mL elution buffer, with 10 μL buffer used for the reaction. The reaction was conducted at 62.5°C for 35 min in a turbidity-measuring real-time device (LoopampEXIA®; Eiken Chemical). A positive result for the LAMP reaction was determined automatically by LoopampEXIA, based on turbidity.

Detection of antibodies against SARS-CoV-2 by enzyme-linked immunosorbent assays (ELISAs)

To measure IgG antibody titers against the N and S antigens of SARS-CoV-2, the QuaResearch COVID-19 Human IgM IgG ELISA Kit (Nucleocapsid Protein) (RCoEL961N; Cellspect Co., Ltd., Iwate, Japan) and the QuaResearch COVID-19 Human IgM IgG ELISA Kit (Spike Protein) (RCoEL961; Cellspect Co., Ltd.) were used, respectively. Serum samples were diluted to 1:1000 for the N antigen ELISA and 1:250 for the S antigen ELISA in 1% bovine serum albumin/PBS with Tween 20 (PBS-T). For the assay, a 100 μL diluted serum sample was added to each well. After incubation at room temperature for 1 h, the plates were washed three times with PBS-T. Specific antibodies were detected using 100 μL horseradish peroxidase-conjugated anti-human IgG at room temperature for 1 h, after which the plates were washed three times with PBS-T. The enzymatic reaction was developed with 100 μL TMB substrate at room temperature for 10 min. Finally, the reaction was stopped using 100 μL of 1 M HCl and the plates were read at 450 nm using
an ELISA reader. The experiments were performed at CellSpect Co., Ltd.

Detection of antibodies against seasonal HCoVs by in-house ELISAs

The S and N proteins of each HCoV (HCoV-HKU1, -OC43, -NL63, and -229E) were obtained from Sino Biological, Inc. (Beijing, China). Details of the antigens used in this study are shown in Supplementary Table 1. Microplates (Corning, Steuben, NY) were coated with 100 μL carbonate-bicarbonate buffer (0.05 M, pH 9.6) containing the S or N antigen of each HCoV (Sino Biological, Inc.) at an optimal concentration of 50 ng/well. After incubation overnight at 4°C, the plates were washed three times with PBS-T and blocked with 200 μL PBS with 1% Block Ace (KAC Co., Ltd., Kyoto, Japan) — a milk protein-based blocking reagent — at 37°C for 1 h. Upon drying overnight at 50°C, the plates were sealed and stored at 4°C until use. For the assay, human serum was diluted 1:100 in PBS containing 1% Block Ace, and 100 μL of the sample was added to each well. After incubation at 37°C for 1 h, the plates were washed three times with PBS-T and specific antibodies were detected using 100 μL horseradish peroxidase-conjugated mouse anti-human IgG (catalog no. 109-035-088; Jackson ImmunoResearch Laboratories, Inc., Sacramento, CA) diluted 1:20 000 in PBS with 1% Block Ace at 37°C for 1 h. The plates were washed three times with PBS-T and the enzymatic reaction was developed with 100 μL per well of solution from a TMB Peroxidase ELISA Substrate Kit (Bio-Rad, Hercules, CA) at 37°C for 30 min. Finally, the reaction was stopped using 100 μL of 1 M H₂SO₄/well and the plates were read at 450 nm using an ELISA reader.

Detection of seasonal HCoV RNA by RT-qPCR

Viral RNA was extracted from nasopharyngeal specimens homogenized in PBS using a QiAsymphony™ RNA Kit (QIAGEN) and High Pure Viral RNA Kit (Roche), following the manufacturers’ instructions. Seasonal HCoV-specific RT-qPCR amplification was performed using a QuantiTect Virus Kit (QIAGEN) with the primers and probes shown in Supplementary Table 2. Thermal cycling conditions consisted of reverse transcription at 50°C for 20 min and 95°C for 5 min, and 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 55°C for 45 s. RT-qPCR samples were analyzed in duplicate; a positive result in both reactions indicated the presence of viral RNA.

Statistical analysis

All serum samples were evaluated by ELISA in triplicate, with the average optical density (OD) value for these measurements defined as the test result. Fold change was determined by measuring the fold change in OD values in IgG antibody recognition between two subsequent paired specimens collected on admission and during convalescence (OD_convalescence/OD_admission). Continuous variables were expressed as mean and standard deviation or median and IQR, and compared using the Student’s t-test or Wilcoxon rank-sum test for parametric or non-parametric data, respectively. Linear regression analysis was used to assess the relationships between each continuous variable. Rho values were analyzed by Pearson’s correlation coefficient and the Spearman rank-order correlation coefficient for parametric or non-parametric data, respectively. A two-sided p-
value < 0.05 was considered statistically significant. All statistical analyses were conducted using R (v 4.0.2; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/).

Results

SARS-CoV-2 antibody titers

In total, 194 paired serum samples from the admission and convalescence phases were collected from 97 patients (mild, 35 patients; severe, 62 patients) and analyzed by ELISAs. Median OD values for SARS-CoV-2 IgG-S (admission 0.04 vs convalescence 0.24; p < 0.001) and IgG-N (admission 0.32 vs convalescence 2.12; p < 0.001) were significantly higher during convalescence than on admission (Figure 1). There were no differences in SARS-CoV-2 IgG-S and IgG-N OD values between the severe and mild cases on admission and during convalescence (Figure 1), whereas fold changes among paired specimens were higher in the severe cases than in the mild cases (severe vs mild, p < 0.001) (Figure 2).

Seasonal HCoV antibody titers in COVID-19 patients

On admission, the prevalence of patients with OD values > 0.5 for IgG-S and/or IgG-N was 82.5% for HCoV-HKU1, 97.9% for HCoV-OC43, 99.0% for HCoV-NL63, and 99.0% for HCoV-229E. The corresponding rates for OD values > 1.0 were 45.4% for HCoV-HKU1, 77.3% for HCoV-OC43, 82.5% for HCoV-NL63, and 75.3% for HCoV-229E (Figure 3). All of the patients showed OD values > 0.5 for IgG-S and/or IgG-N for at least one of the HCoVs. No specific distribution pattern for HCoV seropositivity rate was associated with disease severity (Figure 3).

In the severe cases, median OD values for HCoV-HKU1 IgG-S (admission 0.91 vs convalescence 1.26; p < 0.001), HCoV-OC43 IgG-S (admission 1.13 vs convalescence 1.86; p < 0.001), HCoV-NL63 IgG-N (admission 1.71 vs convalescence 2.34; p = 0.006), and HCoV-229E IgG-N (admission 0.75 vs convalescence 1.21; p = 0.010) were higher during convalescence than on admission, whereas in the mild cases there were no differences between OD values (p > 0.05) (Figure 1). Although there were no differences in OD values for seasonal HCoVs on admission between the severe and mild cases — HCoV-HKU1 IgG-S (mild 0.91 vs severe 1.26; p = 0.025) and HCoV-OC43 IgG-S (mild 1.13 vs severe 1.86; p = 0.027) — OD values were higher in the severe cases than in the mild cases during convalescence (Figure 1). There were no significant differences in fold change among the paired specimens in severe and mild cases (Figure 2). The correlations between overall fold change in antibody levels for seasonal HCoVs and SARS-CoV-2 are shown in Table 1. Regression analysis showed positive correlations for the fold change of OD values for SARS-CoV-2 IgG-S with HCoV-HKU1 IgG-S (Rho = 0.28; p = 0.005) and with HCoV-OC43 IgG-S (Rho = 0.45; p < 0.001). There was no significant correlation between other SARS-CoV-2 and seasonal HCoV antibodies (Table 1).

Effects of clinical characteristics on fold changes of OD values for SARS-CoV-2 and seasonal HCoVs

Regression analysis was performed to evaluate the associations between antibody production and clinical characteristics previously associated with severity and mortality in COVID-19, including age and levels of lactate dehydrogenase (LDH), C-reactive protein (CRP), and lymphocytes. There were positive correlations be-

Figure 2. Comparison of fold change for OD values from admission to convalescence (ODconvalescence/ODadmission) for SARS-CoV-2 and seasonal HCoVs, according to disease severity. The y-axis shows the fold-change level from admission to convalescence for ELISAs. Blue box, mild cases; red box, severe cases; black plots, fold-change levels for each patient; adm, admission; conv, convalescence; *p < 0.05; **p < 0.01; and ***p < 0.001.
between fold change of SARS-CoV-2 IgG-S OD values and age, CRP, and LDH, which were observed at the highest level during hospitalization (p < 0.001), and between those for IgG-N and CRP (p = 0.019). Negative correlations were found between lymphocyte levels, which were observed at the lowest level during admission, and the fold change of SARS-CoV-2 IgG-S (p < 0.001) and IgG-N (p = 0.020) OD values (Figure 4). In addition, positive correlations were observed between the fold change of HCoV-OC43 IgG-S OD values and CRP (Rho = 0.32; p = 0.001) and LDH (Rho = 0.34; p < 0.001) levels, and a negative correlation with lymphocyte levels (Rho = −0.21; p = 0.037), whereas a positive correlation was detected between the fold change of HCoV-HKU1 IgG-S OD values and LDH (Rho = 0.21; p = 0.041) (Figure 4). There was no significant correlation between seasonal HCoV antibody OD values and age. Of the 97 hospitalized patients with COVID-19, nasopharyngeal specimens were collected from 62 patients on admission and tested by RT-qPCR for seasonal HCoVs. However, there was no coinfection between any of the seasonal HCoVs and COVID-19 in the tested specimens (Supplementary Table 3).
Figure 4. Regression analyses between fold-change levels of OD values from admission to convalescence (OD_{convalescence}/OD_{admission}) for SARS-CoV-2 IgG-S and IgG-N, HCoV-HKU1 IgG-S, and HCoV-OC43 IgG-S, and clinical characteristics. The highest levels during hospitalization were observed for CRP and LDH, and the lowest levels were for lymphocyte counts. Regression analyses were conducted using linear regression. Blue plots, mild cases; red plots, severe cases; adm, admission; conv, convalescence; *p < 0.05; **p < 0.01; and ***p < 0.001

Discussion

Our study assessed the cross-reactivity of antibodies for seasonal HCoVs in patients with COVID-19. Our results suggested that the majority of Japanese COVID-19 patients had antibodies to seasonal HCoVs due to previous infections. However, there was no association between the antibody titer for seasonal HCoVs in the early phase of SARS-CoV-2 infection and the severity of COVID-19. Several antibodies for seasonal HCoVs, especially HCoV-OC43 IgG-S, were increased after SARS-CoV-2 infection in the severe cases, whereas there was no significant change in antibodies for seasonal HCoVs in the mild cases. In Japan, seasonal epidemics of HCoVs occur mainly in winter, with HCoV-OC43 detected most frequently in patients with acute respiratory symptoms, followed by HCoV-NL63, -HKU1, and -229E, in 2010–2019 (Komabayashi et al., 2020; Matoba et al., 2015). However, seroprevalence data for seasonal HCoVs in the Japanese population are not available. Our study found that the majority of patients with COVID-19 had antibodies against seasonal HCoVs in the early period after the onset of symptoms, suggesting a high seroprevalence of seasonal HCoVs. Antibodies against HCoV-OC43, -NL63, and -229E were detected most frequently in the patients, which was consistent with the epidemiology of seasonal HCoVs in Japan (Komabayashi et al., 2020). A previous study suggested that the antibody response patterns for SARS-CoV-2 showed second infection patterns in the Japanese population due to cross-reactivity caused by previous infections with seasonal HCoVs (Imai et al., 2021). Our results supported the high seroprevalence of seasonal HCoVs, which could affect the antibody response patterns for SARS-CoV-2.

It remains unclear whether immunity to COVID-19 induced by seasonal HCoV infections leads to cross-protection or disease ex-
acercation. Dugas et al. and Greenbaum et al. showed that high levels of anti-HCoV-OC43 or anti-HCoV-HKU1 S and N antibodies were associated with reductions in severity of COVID-19 or hospital death in studies with small numbers of samples (Dugas et al., 2021; Dugas et al., 2021; Greenbaum et al., 2021). In contrast with the above reports, Ringlander et al. and Sermet-Gaudelus et al. showed that previously verified infections with seasonal HCoVs were not associated with protection against SARS-CoV-2 infection or disease exacerbation in adults or children, respectively (Ringlander et al., 2021; Sermet-Gaudelus et al., 2021). Indeed, our study showed no association between the antibody titers of seasonal HCoVs and disease severity in the early phase of COVID-19. Our results agree with the findings that humoral immunity induced by seasonal HCoV infections may not prevent disease exacerbation in COVID-19.

Interestingly, our study found that the OD values for HCoV-HKU1 and -OC43 IgG-S, which are betacoronaviruses, as well as for SARS-CoV-2 IgG-S and IgG-N, were significantly higher in the severe cases than in the mild cases during convalescence. This phenomenon was also reported by Wang et al., 2020a); however, it has not been investigated whether the elevation of antibodies occurs before or after infection with SARS-CoV-2. Our study showed that the fold change of antibody OD values occurred after SARS-CoV-2 infection, and was associated with disease severity. There was a positive correlation between SARS-CoV-2 and HCoV-OC43 IgG-S in terms of the magnitude of the change of OD values. SARS-CoV-2 and HCoV-OC43 IgG-S OD values were also correlated with CRP, LDH, and lymphocyte levels, which are reported to be associated with disease severity. Ladner et al. showed that antibodies for SARS-CoV-2 cross-recognize seasonal HCoV antigens at two highly conserved regions of the S2 subunit epitope (Ladner et al., 2021). It is assumed that the excess production of cross-reactive antibodies for the highly conserved regions of S2 of other betacoronaviruses is induced by SARS-CoV-2 infection in severe COVID-19 cases. One of the concerns is that the excess production of cross-reactive antibodies for the S antigen of seasonal HCoVs may be partly responsible for disease exacerbation in patients with COVID-19. In several viral infections, including SARS-CoV-1, excess antibody production can lead to disease exacerbation via antibody-dependent enhancement (Katzelnick et al., 2017; Wang et al., 2014; Yip et al., 2014). Wang et al. and Wu et al. showed that, in addition to SARS-CoV-1, an antibody targeting the S protein of SARS-CoV-2 could cause antibody-dependent enhancement in COVID-19 (Wang et al., 2020b; Wu et al., 2020). In vitro and in vivo experiments using IgG-S, especially antibodies for S2, of seasonal HCoVs should be performed to examine the possibility of antibody-dependent enhancement by those antibodies.

Our study presented some limitations. The serum samples used were collected during the early phase of COVID-19 symptom onset, and not before SARS-CoV-2 infection. Therefore, the humoral immunity for seasonal HCoVs may not have been an exact representation of humoral immunity at the time of SARS-CoV-2 infection. Only ELISAs were used to detect the antibodies for seasonal HCoVs; it remains possible that the antibody response patterns are assay-specific. Furthermore, the serum samples were collected only from Japanese patients; this may have introduced selection bias, because the seroprevalence rates for seasonal HCoVs vary among regions and countries.

Our study did not investigate the cross-reactivity of other structural proteins (E and M), non-structural proteins, accessory proteins, IgM and IgA antibodies, and the T-cell response. Several studies have shown that 20–60% of people who have not been infected with SARS-CoV-2 have T-cell reactivity against peptides corresponding to SARS-CoV-2 sequences, including S, N, M, and non-structural proteins, and open reading frames (Braun et al., 2020; Grifoni et al., 2020; Le Bert et al., 2020; Meckiff et al., 2020; Weiskopf et al., 2020). In addition, Mateus et al. found that SARS-CoV-2 peptides with > 67% homology with the corresponding peptides of seasonal HCoVs were associated with the cross-reactivity of CD4+ T cells (Mateus et al., 2020). These findings suggest that pre-existing cross-reactive HCoV CD4+ memory T cells in COVID-19 patients could be a contributing factor to variations in disease outcome.

Additional large-scale and multinational investigations, combining the evaluation of humoral and T cell responses, are required to determine whether cross-reactive humoral immunity against seasonal HCoVs has a positive or negative impact on the severity of COVID-19.

Conclusion

Our study showed that there was no association between the antibody titers of seasonal HCoVs during the early phase of COVID-19 and disease severity. Significantly increased OD values for HCoV-HKU1 and -OC43 IgG-S from early symptom onset to the convalescence period were observed in severe cases, but not in mild cases. Further investigations are required to determine whether cross-reactivity between SARS-CoV-2 and seasonal HCoV antibodies is associated with the severity of COVID-19.

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Author contributions

YS and TM conceived and designed the study. KI, SaT, MNL, and MK collected data and performed data analysis. KI, MM, YK, KK AF TT, MS, and SN collected specimens and performed experiments. KI and TM drafted and edited the manuscript. NA, YS, and SHT revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors report no conflicts of interest relevant to the published work.

Hammitt et al., 2011

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