Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CLINICAL INVESTIGATION

Serum cystatin C and CRP are early predictive biomarkers for emergence of hypoxia in COVID-19

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ABSTRACT

Background: In Japan, during the coronavirus disease 2019 (COVID-19) pandemic, patients with non-hypoxia are recommended to recuperate at home or in pre-hospital facilities. However, it was observed that unexpected hypoxia may occur and become severe subsequently in patients whose symptoms were initially expected to improve naturally. The aim of this study is to validate biomarkers that can predict at an early stage the emergence of hypoxia in COVID-19 patients without hypoxia.

Methods: We retrospectively enrolled 193 patients with COVID-19, excluding patients with hypoxia and severe disease from the onset. Participants were classified into two groups according to the emergence of hypoxia during the clinical course, and the laboratory data were compared to identify biomarkers that could predict early the emergence of hypoxia.

Results: The areas under the curve for serum cystatin C (CysC) and C-reactive protein (CRP) levels for the emergence of hypoxia during the clinical course were higher than those for other biomarkers (CysC, 0.84 and CRP, 0.83). Multivariate analysis showed that high serum CysC and CRP levels were associated with the emergence of hypoxia during the clinical course.

Conclusions: Elevated serum CysC and CRP levels were associated with the emergence of hypoxia during the clinical course in COVID-19 patients without hypoxia. These findings may help determine the need for hospitalization in initially non-hypoxic COVID-19 patients.

Key Indexing Terms: COVID-19; Cystatin C; C-reactive protein; Hypoxia; RT-PCR. **[Am J Med Sci 2022;■(■):1–8.]**

INTRODUCTION

Coronavirus disease 2019 (COVID-19) cases are increasing exponentially. As of April 22, 2021, over 140 million people have been infected and over 3 million people have died worldwide.[1] Japan experienced three major waves of a surge in infection between 2020 and 2021, creating social problems.[2]

In Japan, recuperation at home or in a hotel is recommended for patients with low disease severity during surges in infection. However, some patients who were convalescing at home or in pre-hospital facilities because they did not need oxygen treatment experienced rapid hypoxia during the clinical course, requiring hospitalization or even resulting in death.[3] Therefore, it is necessary to elucidate the factors that can predict the

emergence of hypoxia, even in non-hypoxic COVID-19 patients.

Various factors that predict disease severity, such as oxygen demand and in-hospital mortality, have been reported. A Japanese study involving 345 COVID-19 patients reported that oxygen demand is associated with age, shortness of breath, and general fatigue, and death is associated with age, hyperuricemia, and chronic kidney disease.[4] In addition, data from patients with acute hypoxic respiratory failure in New York showed that age, chronic cardiac disease, chronic pulmonary disease, interleukin (IL)-6 and D-dimer levels are associated with in-hospital mortality.[5]

However, there are still few reports on biomarkers that can predict the emergence of hypoxia, validated only in COVID-19 patients without hypoxia. An investigation of these biomarkers may identify the criteria for recommending hospitalization, even in patients who are not hypoxic and whose symptoms are expected to improve naturally. The number of hospital beds is limited universally, and hence, it is important to identify those patients who should be recommended hospitalization during drastic surges in infection. Therefore, in this study, we validated the biomarkers that may predict the emergence of hypoxia during the COVID-19 clinical course in patients who did not require oxygen treatment.

METHODS

Study design and patients

In this double-center retrospective study, we investigated the biomarkers that could predict the emergence of hypoxia early during the clinical course in COVID-19 patients without hypoxia. We enrolled 281 COVID-19 patients admitted to the Showa University Hospital and Showa University East Hospital from March 28, 2020 to April 22, 2021. Of the 88 excluded patients, 28 were transferred from or to another hospital during the disease course, 23 required oxygen treatment at admission, 11 were administered COVID-19 therapeutic agents (remdesivir, favipiravir, tocilizumab, baricitinib, and/or steroids) and discharged without oxygen treatment, 14 did not undergo chest computed tomography (CT), five were suspected of having sleep apnea and were administered oxygen during sleep, six had incomplete data, and one patient had initiated home oxygen therapy before admission (Figure 1).

We analyzed 193 patients who satisfied the inclusion criteria. First, we categorized the patients into two groups: one with patients that were discharged without hypoxia during the clinical course ("non-hypoxia group") and one in which oxygen treatment was administered due to hypoxia ("hypoxia group"). Hypoxia was defined as the condition at which oxygen treatment was started

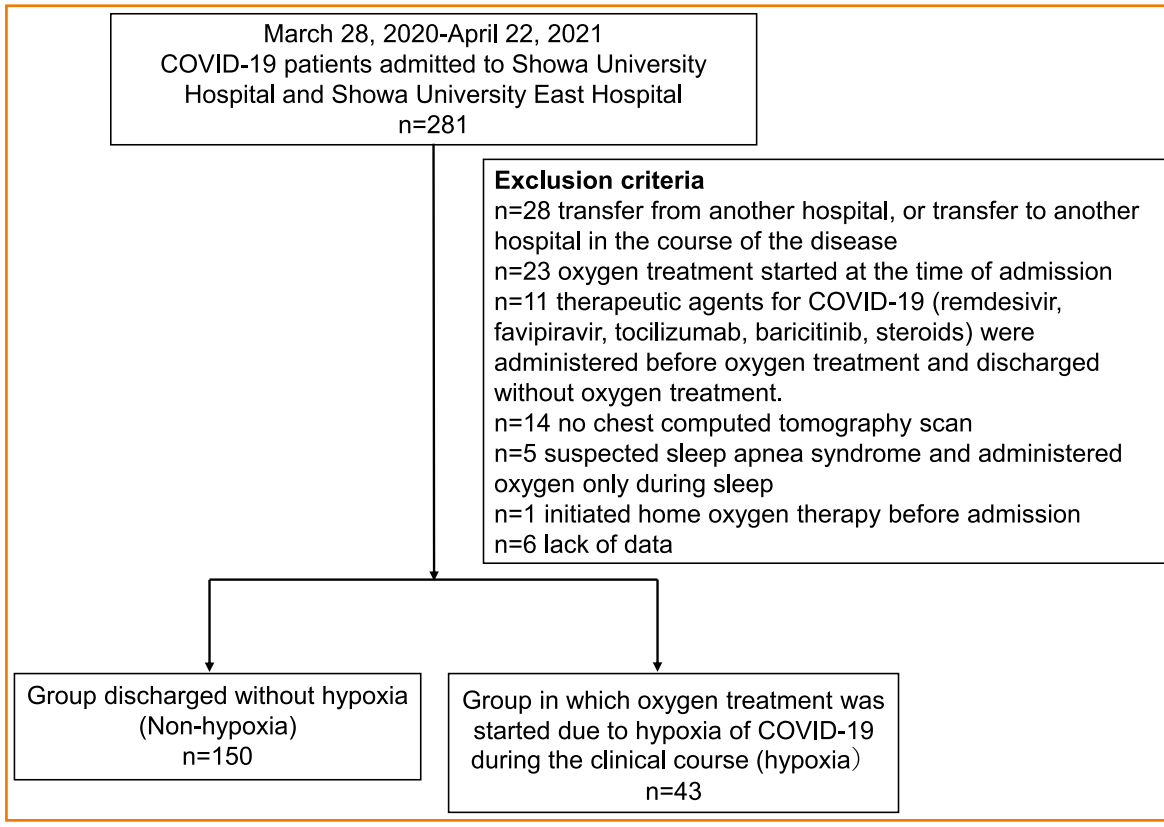

FIGURE 1. Consolidated Standards of Reporting Trials flow diagram. COVID-19: Coronavirus disease 2019.

with percutaneous oxygen saturation <90%. Second, patient characteristics, comorbidities, symptoms, and laboratory data were compared between the two groups. All laboratory data were obtained within 24 hours before and after admission with a diagnosis of COVID-19. We also compared the threshold cycle (Ct) values in real-time reverse transcription-polymerase chain reaction (RT-PCR) for COVID-19 between the groups. This study was approved by the ethics committee of the Showa University (Approval number: 3438).

Serum CysC and CRP measurements

Serum cystatin C (CysC) levels were measured using latex agglutination turbidimetric immunoassay by BML (Kawagoe, Japan). Serum C-reactive protein (CRP) levels were measured using latex agglutination turbidimetric immunoassay at the hospital laboratory of Showa University.

RT-PCR measurements

We used the reagent of the SARS-CoV-2 Detection Kit (TOYOBO, OSAKA, Japan) and performed RT-PCR on nasopharyngeal swab samples at the Showa University Hospital PCR center for COVID-19. The RT-PCR assays were conducted in a QuantStudio 3 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). A total of 45 cycles were set with the following cycling conditions: 42°C for 5 min for reverse transcription, 95°C for 10 s for pre-denaturation, 95°C for 1 s for denaturation, 50°C for 10 s for annealing, and 55°C for 10 s for extension. All Ct values obtained in this study were measured using a N2 primer-probe set.

Statistical analysis

The analyses were performed using JMP Pro ® 15 (SAS Institute Inc., Cary, NC, USA). The Anderson-Darling test showed that no continuous variables were normally distributed. Continuous variables are expressed as median (interquartile range [IQR]), and statistical significance was set at $p < 0.05$ (two-sided). Differences between the groups were analyzed with χ_2 test for categorical variables and unpaired Mann-Whitney U-test for continuous variables.

Moreover, we considered the emergence of hypoxia during the clinical course as the dependent variable, created receiver operating characteristic (ROC) curves, calculated areas under the curves (AUCs), and measured the cutoff values for white blood cell (WBC), D-dimer, creatinine, lactate dehydrogenase (LDH), ferritin, CRP, and CysC levels. Optimal cutoff values were determined using the Youden index.

Additionally, we performed multivariate logistic regression analysis with the emergence of hypoxia during the clinical course as the dependent variable and age, gender, pneumonia, CRP, and CysC as independent variables.

RESULTS

Patient characteristics

The median observation period in the hospital was 10 (IQR, 8-12) days. Table 1 shows the patient characteristics. The study population comprised 100 men (51.8%) and 93 women (48.2%), and the median age was 47 (IQR, 30-60) years. The median body mass index (BMI) was 22.7 kg/m^2, and 18 (9.3%) patients were obese (BMI $\geq$ 30 kg/m^2). Hypertension was the most common comorbidity (20.2%), followed by hyperlipidemia (13.5%), diabetes (6.7%), and emphysema (6.7%). The most common symptoms were fever (79.3%), followed by cough (45.1%) and olfactory disorder (22.8%). Additionally, 67 (34.7%) patients reported a history of smoking and 117 (60.6%) exhibited pneumonia on CT.

Comparison between hypoxia and non-hypoxia groups

Table 1 shows the results of comparison between the groups. Among patient characteristics, there were significant differences in age, sex, BMI, and smoking history between the two groups. Moreover, significant differences were evident in findings suggestive of pneumonia on chest CT and in the symptom of sore throat. Among comorbidities, there were significant differences in the incidence of hypertension, diabetes, hyperlipidemia, hyperuricemia, emphysema, and chronic obstructive pulmonary disease (COPD). Furthermore, the laboratory data showed significant differences in WBC, D-dimer, creatinine, LDH, CRP, ferritin, and CysC levels.

Early predictive factors for need of oxygen treatment

The results of multivariate logistic regression analysis showed that older age (hazards ratio [HR], 1.60 [95% CI 1.08-2.46] per 10-year increase), presence of pneumonia (HR, 10.40 [95% CI 1.63-186.13]), high CRP (HR 1.39 [95% CI 1.10-1.85]), and high CysC (HR 1.36 [95% CI 1.09-1.82] per 0.1 mg/L increase) were associated with the emergence of hypoxia during the clinical course (Table 2).

Supplementary Figure 1 shows the Ct values in RT-PCR for all patients. The median Ct value for the entire study sample was 28.6 (IQR 23.1-32.4) cycles, and the median time from disease onset to data collection was 6 (IQR 2-10) days. As shown in Supplementary Figure 1A, 132 patients who underwent RT-PCR at least once during hospitalization and in whom Ct values were measured were categorized into 25 and 107 patients in the hypoxia and non-hypoxia groups, respectively. Moreover, the Ct value in RT-PCR for COVID-19 is thought to attain a peak within one week of onset.[6] With reference to this report, 78 patients who underwent RT-PCR at our hospital within one week of onset were categorized into 13 and 65 patients in the hypoxia and non-hypoxia groups, respectively (Supplementary Figure 1B). The two groups were compared using the first Ct values

Table 1. Baseline clinical characteristics and laboratory data of patients included in the study.

	Total (*N* = 193)	Non-hypoxia (*N* = 150)	Hypoxia (*N* = 43)	*P*- value
Age (years)				
Median	47 (30-60)	41 (27-55)	65 (53-76)	<0.0001
10-19	3 (1.6%)	3 (2.0%)	0 (0.0%)	
20-29	43 (22.3%)	43 (28.6%)	0 (0.0%)	
30-39	30 (15.5%)	28 (18.7%)	2 (4.7%)	
40-49	28 (14.5%)	23 (15.3%)	5 (11.6%)	
50-59	40 (20.7%)	30 (20.0%)	10 (23.3%)	
60-69	15 (7.8%)	7 (4.7%)	8 (18.6%)	
70-79	25 (13.0%)	12 (8.0%)	13 (30.2%)	
80-89	8 (4.1%)	4 (2.7%)	4 (9.3%)	
90-99	1 (0.5%)	0 (0.0%)	1 (2.3%)	
Sex				
Female	93 (48.2%)	80 (53.3%)	13 (30.2%)	0.0092
Male	100 (51.8%)	70 (46.7%)	30 (69.8%)	
Race				
Japanese	183 (94.8%)	143 (95.3%)	40 (93.0%)	0.6955
Non-Japanese	10 (5.2%)	7 (4.7%)	3 (7.0%)	
BMI (kg/m^2)	22.7 (20.0-25.1)	21.8 (19.8-24.6)	24.0 (22.8-27.0)	0.0002
Obesity	18 (9.3%)	11 (7.3%)	7 (16.3%)	0.1316
Smoking history				
Current or former smoker	67 (34.7%)	43 (28.7%)	24 (55.8%)	0.0018
Finding by CT				
Pneumonia	117 (60.6%)	76 (50.7%)	41 (95.3%)	<0.0001
Respiratory support				
Nasal cannula oxygen therapy	37 (19.2%)	0	37 (86.0%)	<0.0001
Face mask oxygen therapy	3 (1.6%)	0	3 (7.0%)	
IMV	3 (1.6%)	0	3 (7.0%)	
Symptoms				
Fever	153 (79.3%)	120 (80.0%)	33 (76.7%)	0.6715
Cough	87 (45.1%)	69 (46.0%)	18 (41.9%)	0.7287
Sputum	16 (8.3%)	14 (9.3%)	2 (4.7%)	0.5308
Sore throat	49 (25.4%)	44 (29.3%)	5 (11.6%)	0.0178
Rhinorrhea	20 (10.4%)	15 (10.0%)	5 (11.6%)	0.7783
Dyspnea	25 (13.1%)	22 (14.7%)	3 (7.0%)	0.3011
Taste disorder	43 (22.3%)	37 (24.7%)	6 (14.0%)	0.1518
Olfactory disorder	44 (22.8%)	36 (24.0%)	8 (18.6%)	0.54
Nausea	8 (4.1%)	6 (4.0%)	2 (4.7%)	1.0000
Diarrhea	11 (5.7%)	7 (4.7%)	4 (9.3%)	0.2672
Comorbidities				
Hypertension	39 (20.2%)	20 (13.3%)	19 (44.2%)	<0.0001
Diabetes	13 (6.7%)	8 (5.3%)	5 (11.6%)	0.1688
Hyperlipidemia	26 (13.5%)	16 (10.7%)	10 (23.3%)	0.0430
Hyperuricemia	10 (5.2%)	4 (2.7%)	6 (14.0%)	0.0092
Bronchial asthma	11 (5.7%)	8 (5.3%)	3 (7.0%)	0.7112
Emphysema	13 (6.7%)	3 (2.0%)	10 (23.3%)	<0.0001
COPD	2 (1.0%)	0 (0%)	2 (4.7%)	0.0487
Sleep apnea syndrome	5 (2.6%)	2 (1.3%)	3 (7.0%)	0.0743
Laboratory data				
White blood cell count (/ μl)	4500 (3700-5550)	4350 (3600-5300)	5100 (4200-6200)	0.0020
Lymphocytes count (/ ml)	1040 (780-1355)	1080 (790-1370)	950 (770-1250)	0.2959
D-dimmer (mg/ml)	0.74 (0.59-0.98)	0.70 (0.56-0.88)	0.96 (0.78-1.17)	<0.0001
Creatinine (mmol/L)	0.7 (0.57-0.85)	0.65 (0.55-0.82)	0.82 (0.70-0.96)	<0.0001
Lactate dehydrogenase (U/L)	189 (161-233)	176 (155-215)	241 (214-301)	<0.0001
Creatine kinase (U/L)	74 (51-119)	74 (50-117)	75 (55-149)	0.3520
C-reactive protein (mg/dl)	0.89 (0.24-2.78)	0.51 (0.19-2.05)	4.20 (1.44-7.83)	<0.0001
Ferritin (ng/mL)	207 (87-505)	171 (66-361)	598 (229-893)	<0.0001
Cystatin C (mg/L)*	0.87 (0.75-0.99)	0.83 (0.73-0.92)	1.04 (0.93-1.31)	<0.0001

Data are expressed as median (interquartile range) or N (%).

BMI, body mass index; COPD, Chronic obstructive pulmonary disease; IMV, Invasive mechanical ventilation

* Cystatin C was measured in a total of 152 patients, 116 in the non-hypoxia group and 36 in the hypoxia group.

Table 2. Logistic regression analysis for the emergence of hypoxia in COVID-19 patients without hypoxia.

Risk factors	Odds ratio	95% CI	*P*- value
Age (per 10-year increase)	1.60	1.08-2.46	0.0219
Sex (Male)	3.05	0.90-11.66	0.0835
Pneumonia	10.40	1.63-186.13	0.0397
C-reactive protein	1.39	1.10-1.85	0.0145
Cystatin C (per 0.1mg/L increase)	1.36	1.09-1.82	0.0146

95% CI; 95% confidence interval

Table 3. Cutoff, area under the curve, and *P* values for each biomarker.

	cutoff value	AUC (CI)	*P*- value
Cystatin C (mg/L)	0.97	0.84 (0.75-0.90)	<0.0001
C-reactive protein (mg/dl)	2.75	0.83 (0.75-0.89)	<0.0001
Lactate dehydrogenase (U/L)	196	0.82 (0.74-0.88)	<0.0001
Ferritin (ng/mL)	482	0.77 (0.69-0.84)	<0.0001
D-dimmer (mg/ml)	0.76	0.75 (0.67-0.82)	<0.0001
Creatinine (mmol/L)	0.68	0.74 (0.65-0.81)	<0.0001
White blood cell count (/μl)	5700	0.65 (0.55-0.74)	0.0063

measured at our hospital, and no significant differences were observed.

AUC and cutoff value for each biomarker

Figure 2 and Table 3 show the ROC curve and AUC for each biomarker. The AUCs for CysC, CRP, LDH, ferritin, D-dimer, creatinine, and WBC levels were 0.84 (95% confidence interval [CI] 0.74-0.89), 0.83 (95% CI 0.74-0.89), 0.82 (95% CI 0.73-0.87), 0.77 (95% CI 0.69-0.85), 0.75 (95% CI 0.66-0.82), 0.74 (95% CI 0.65-0.81) and 0.65 (95% CI 0.54-0.73), respectively. Additionally, the cutoff values for CysC, CRP, LDH, ferritin, D-dimer, creatinine, and WBC levels were 0.97 mg/L, 2.75 mg/dL, 196 U/L, 482 ng/mL, 0.76 mg/ml, 0.68 mmol/L, and 5700 /μl, respectively.

The sensitivity, specificity, positive predictive value, and negative predictive value of CysC were 0.69, 0.86, 0.58, and 0.90, respectively, while those for CRP were 0.75, 0.82, 0.57, and 0.91, respectively. Moreover, the sensitivity, specificity, positive predictive value, and negative predictive value when both CRP and CysC levels were considered concomitantly were calculated for 152 patients. When CRP ≥2.57 mg/dL and CysC level ≥0.97 mg/L, these values were 0.55, 0.95, 0.8, and 0.87, respectively, and when CRP <2.57 mg/dL and CysC <0.97 mg/L, they were 0.86, 0.74, 0.50, and 0.94, respectively. In other words, 80% cases exceeding the cutoff values for both biomarkers were in the hypoxia group, and 94% cases not exceeding the cutoff values were in the non-hypoxia group (Figure 3).

Furthermore, we investigated the relationship between CRP and CysC levels and the number of days from disease onset to laboratory investigations. We categorized 39 patients in the hypoxia group with known dates of disease onset (for asymptomatic patients, the PCR positive date was considered the onset date) into 26 patients with CRP ≥2.75 mg/dL and 13 patients with CRP <2.75 mg/dL, and

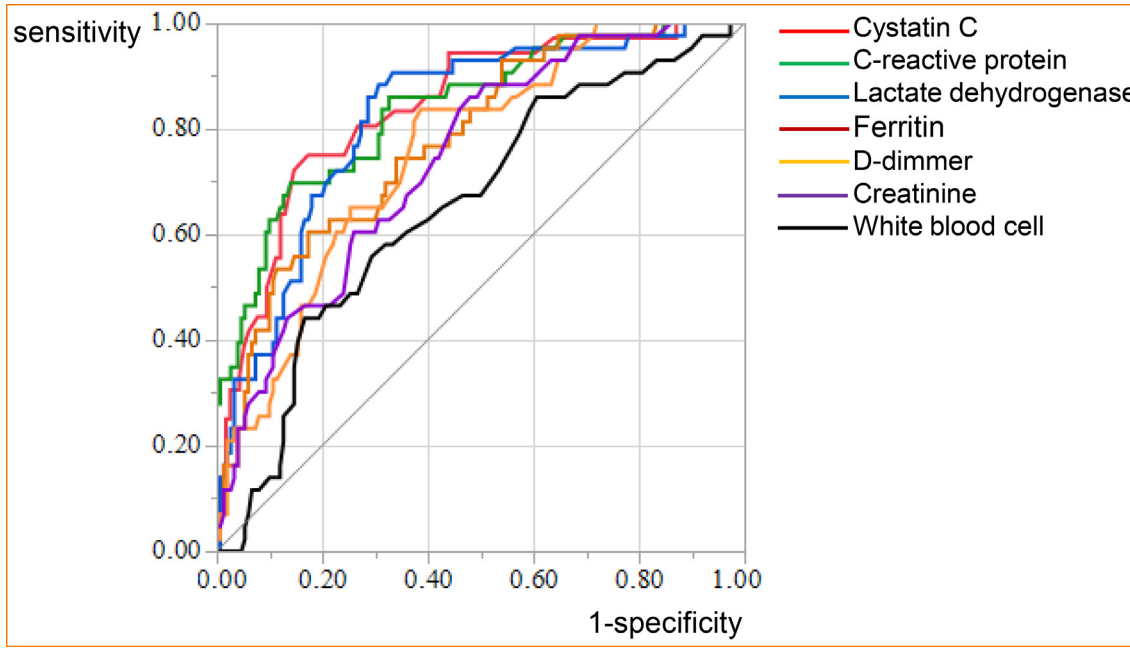

FIGURE 2. Receiver operating characteristic curve for each biomarker pertaining to the emergence of hypoxia during the clinical course.

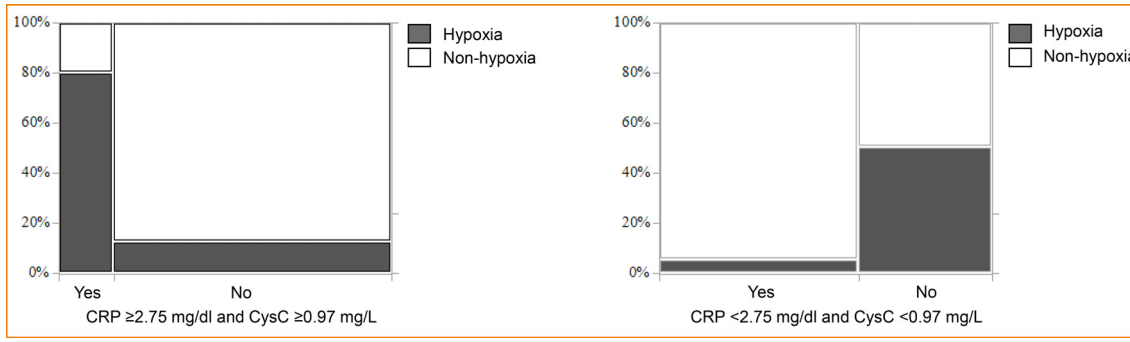

FIGURE 3. Frequency of patients with hypoxia of COVID-19 according to serum CRP and CysC levels. Hypoxia group: the group in which oxygen treatment was required in patients because of hypoxia during the clinical course Non-hypoxia group: the group in which patients were discharged without hypoxia during the clinical course. COVID-19: Coronavirus disease 2019; CRP: C-reactive protein; CysC: Cystatin C

compared the number of days from onset to data collection. For CysC, we categorized 34 patients in hypoxia group with known onset dates into 25 patients with CysC ≥0.97 mg/L and nine patients with CysC <0.97 mg/L, and compared the number of days from onset to data collection (Supplementary Table 1). The median number of days was six for patients with CRP ≥2.75 mg/dL and three for patients with CRP <2.75 mg/dL; the difference was statistically significant (p = 0.0003). However, the median number of days was five for both patients with CysC ≥0.97 mg/L and patients with CysC <0.97 mg/L, and the difference was not statistically significant (p = 0.8586).

DISCUSSION

Our study showed two important findings. First, Serum CysC and CRP levels in COVID-19 patients without hypoxia were associated with the emergence of hypoxia during the clinical course. Second, the cut-off values for serum cystatin C and CRP were 0.97 mg/L and 2.75 mg/dL, respectively. The strength of this study is that it excluded severe cases who required oxygen treatment and only validated the study in a group of patients without hypoxia. This may have clarified the criteria for recommending hospitalization, even for initially patients who are not hypoxic and whose symptoms are expected to improve naturally.

Serum CysC has recently been determined a marker for evaluating renal function decline. It has high sensitivity and is not usually affected by age, sex, body weight, and inflammation.[7] It is also considered a predictor of death from heart failure and cardiovascular disease.[8] Serum CysC is a 13 kDa protein consisting of 122 amino acids and is a cysteine proteinase inhibitor.[9] It suppresses the activation of cysteine protease, an enzyme used by the invading pathogens for proliferation, extracellularly.[10] An increase in serum CysC is also observed in human immunodeficiency virus infection,[11] which may reflect the in-vivo reactions associated with viral infections. Furthermore, serum CysC level is significantly correlated with inflammatory cytokines IL-6 and Tumor necrosis factor (TNF)-α levels,[12] and cytokines from inflammatory cells regulate the expression of serum CysC.[13] COVID-19 is often exacerbated by the release of excess cytokines ("cytokine storm"), which can cause excessive inflammation and multiple organ failure.[14] Therefore, inflammatory cytokines that cause cytokine storm are involved in the expression of serum CysC, as reflected in our research.

In this study, elevated serum CRP levels were associated with the emergence of hypoxia in COVID-19 patients without hypoxia. Serum CRP levels have also been reported to be significantly associated with the increased possibility of ICU hospitalization, intubation, and mortality.[15] Serum CRP level is largely reflected in the exacerbation of COVID-19, which may be due to its association with IL-6. It has been considered that the activation of IL-6 amplifier by COVID-19 specific angiotensin 2-angiotensin receptor type 1, which is a mechanism of IL-6 overproduction for cytokine storm induction, induces inflammatory cytokines.[16] Reflecting this, IL-6 is elevated in patients with severe COVID-19 symptoms,[17] and hence, tocilizumab, a neutralizing antibody against the IL-6 receptor, is now being used as treatment. Elevated IL-6 is significantly associated with the severity of COVID-19. Serum CRP is produced by the action of inflammatory cytokines such as TNF-α, IL-1, and IL-6 produced by macrophages, monocytes, and T cells by lipopolysaccharide stimulation due to infection.[18] Therefore, an elevated serum CRP may mean an elevated IL-6 acting on it. In fact, CRP has been normalized in rheumatoid arthritis and Castleman's disease by treatment that inhibits IL-6.[19,20] The greatest benefit of serum CRP over IL-6 is that it is a biomarker which can be easily and quickly measured at hospital laboratories. However, the problem is with CRP that it is not elevated in the early stages of infection, as also seen in our study (Supplementary Table 1), meaning that if laboratory investigations are performed exceedingly early in the course of the disease, hypoxia may be emerged, even if serum CRP is below the cutoff value.

Unlike previous reports,[21,22] the multivariate analysis showed no significant difference in need for oxygen treatment during the clinical course in men (p = 0.0835), which may be due to COPD and the exclusion of severe disease.

Previous reports have shown that smoking itself is associated with death and hospitalization in COVID-19.[23] Moreover, COPD is associated with an increased risk of death from COVID-19 in men, and a five-fold increased risk of severe COVID-19 symptoms compared to those without any underlying disease.[24,25] However, studies that exclude patients with severe symptoms, such as this study, may exclude COPD patients, which could result in lesser impact of sex. In fact, the proportion of men and COPD patients in this study was even lower than in studies including patients with severe symptoms.[5]

Unfortunately, there was no significant difference in Ct values between the two groups in our study. Several previous reports have shown a correlation between Ct values in RT-PCR of COVID-19 and mortality.[26,27,28] However, the association of the Ct value with disease severity is controversial, with variable opinions. The lack of significant differences in this study may be attributed to the fact that there were few patients with severe symptoms, such as those who required oxygen significantly and those who died. Moreover, the number of patients who underwent RT-PCR within one week after the onset was small, necessitating further research into the utilization of Ct value.

Our study has several limitations. First, the severity of COVID-19 is affected by several host factors, such as gene mutations and race.[29,30] As approximately 5% of patients in this study population were of non-Japanese descent and ethnic differences were not considered in the analysis, further research is required. Second, a variant of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from the UK was reported in December 2020,[31] which may have been partially included in this study. The recent SARS-CoV-2 variant is associated with a 1.64 times higher risk of death than the original strain.[32] Therefore, it is necessary to evaluate variant-specific effects in the future. Third, serum CysC and CRP levels were included in the multivariate logistic regression analysis to identify biomarkers, but changes in concentration over time were not evaluated. The levels of these markers can change significantly over time, and their effects should be further investigated.

CONCLUSIONS

In conclusion, our findings showed that high serum CysC and CRP in COVID-19 patients without hypoxia was associated with the emergence of hypoxia during the clinical course. The cutoff values were 0.97 mg/L for serum CysC and 2.57 mg/dL for serum CRP. These findings may help in determining the need for hospitalization of COVID-19 patients without hypoxia.

AUTHORS CONTRIBUTIONS STATEMENT

Y.M. designed the study, wrote and revised the paper; H I wrote and revised the paper; K.H. collected and analysed the data; F.I., S.O., H.S., K.M., T.E., H.M., T.K., Y.F., Y.K., T.H., H.O., S.K., M.Y., S.S., Y.U., A.T., K.I. and Y.K. collected data; H.S. supervised and reviewed the manuscript; all authors provided critical review of the manuscript and approved the final draft for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest associated with this manuscript.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.amjms.2022.06.027.

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