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Research Article

Do Levels of the Platelet Activation Markers sCD40 L and SCUBE 1 Differ between Laboratory-Confirmed and Clinically Diagnosed COVID-19 Patients?

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Abstract

Aim: Thromboembolic complications are an important cause of mortality and morbidity in coronavirus disease 2019 (COVID-19) patients. The purpose of this study was to compare levels of the platelet activation markers soluble CD40 ligand (sCD 40L) and signal peptide-CUB-EGF domain-containing protein 1 (SCUBE 1) and hematological parameters between laboratory-confirmed and clinically diagnosed COVID-19 patients. No previous studies have investigated levels of these markers in laboratory-confirmed and clinically diagnosed COVID-19 patients.

Material and Method: Fifty-one laboratory-confirmed and clinically diagnosed COVID-19 patients with no exclusion criteria were enrolled in the study. Blood specimens were collected for SCUBE1, sCD40 L, and hematological and biochemical parameter measurement. These parameters from laboratory-confirmed and clinically diagnosed COVID-19 patients were then compared.

Results: SCUBE1 and sCD40L levels were significantly higher in the laboratory-confirmed group compared to the clinically diagnosed group ($p<0.05$ and $p = 0.005$, respectively). Time elapsing between onset of symptoms and presentation to hospital was significantly shorter in the laboratory-confirmed group, while rates of contact with COVID-19 patients were significantly higher ($p < 0.001$ and $p < 0.005$, respectively). SCUBE 1 levels were significantly negatively correlated with ferritin and C-reactive protein (CRP) ($p< 0.05$, $r= -0.322$ and $p< 0.05$, $r= -0.351$, respectively).

Conclusion: This study shows, for the first time in the literature, that levels of the platelet activation markers SCUBE1 and sCD40L are significantly higher in laboratory-confirmed COVID-19 patients compared to clinically diagnosed individuals.

Keywords: Clinically diagnosed COVID-19; Laboratory-confirmed COVID-19; SCUBE 1; Platelet activation; Coagulation

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly infectious pathogen leading to coronavirus disease 2019 (COVID-19) and high mortality rates. COVID-19 therefore rapidly developed into a pandemic after its first appearance, causing significant morbidity and mortality worldwide [1-3]. Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) is the most widespread and only direct method for determining SARS-CoV-2 for diagnosis of COVID-19. However, laboratory errors, specimen collection at different stages of the disease, and inappropriate or insufficient specimen collection make RT-qPCR inadequate for the diagnosis of COVID-19, and some patients may be diagnosed and treated late. The detection of typical findings of lung involvement with imaging techniques and various laboratory tests is therefore diagnostic of COVID-19 in individuals with symptoms of histories of high-risk contact [4-6].

SARS-CoV-2 is a single-stranded RNA virus that enters human cells by attaching to angiotensin-converting enzyme 2 (ACE 2). ACE 2 receptors are expressed in lung, cardiac myocyte, vascular endothelial, platelet, and other cells [3,7]. After entering the cell via these receptors, the virus causes a series of reactions, particularly cytokine release syndrome, and results in cardiovascular collapse and thromboembolic complications [8]. Hypercoagulability has been reported as the major pathological event in COVID-19 disease, and thromboembolic events are listed as some of the most important life-threatening complications of the disease. Platelets are the main effector cells of hemostasis and pathological thrombus. However, the contribution of platelets to the pathogenesis of COVID-19 remains unclear [9-11].

Platelet activation is evaluated by morphology, function, and measuring the levels of various plasma markers [12]. One such activation marker is soluble CD40 ligand (sCD40L). CD40 L is a transmembrane protein structurally associated with the tumor necrosis factor-α (TNF α) family and is expressed on the cell surface by activated platelets [13]. sCD40L is the form of CD40 L released into plasma from the activated platelet surface. More than 95% of

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sCD40L in plasma is known to originate from platelets [14].

Signal peptide-CUB-EGF domain-containing protein 1 (SCUBE 1) is a cell surface protein and member of the SCUBE gene family [15]. The SCUBE gene family contains three separate isoforms (SCUBE 1-3). SCUBE 1 is expressed in tissues that grow rapidly during embryological development and has been shown in recent years to be secreted from the endothelium and platelets [15,16]. Various studies have shown that SCUBE 1 is stored in platelet a-granules and moves to the cell surface with platelet stimulation and activation [17].

The purpose of the present study was to compare levels of the platelet activation markers SCUBE 1 and sCD40L in laboratory-confirmed (RT-qPCR +) and clinically diagnosed COVID-19 patients and to determine their association with hematological parameters. This is the first study on the subject to date.

Material and Method

Fifty-one patients aged over 18 presenting to the Tekirdağ Namık Kemal University Medical Faculty, Turkey, 27 with laboratory-confirmed (RT-qPCR +) COVID-19 and 24 patients clinically diagnosed with COVID-19 based on chest Computerized Tomography (CT) and clinical findings and started on treatment were included in the study. Patients with hospitalized with the suspicion or diagnosis of COVID-19 before, pregnant women, patients using oral anticoagulant or antiplatelet therapy, with liver failure, or with other systemic infections were excluded. Patients were enrolled once the local ethical committee had confirmed that the study protocol was compatible with the second Declaration of Helsinki. Patients underwent detailed physical examinations, and their demographic data and vital findings were recorded. Nasopharyngeal swab specimens were collected from all patients for the COVID-19 RT-qPCR test. Repeat RT-qPCR specimens were collected after 48 h from patients with negative swab results. Chest CT were performed for the diagnosis of COVID-19 pneumonia. Other viral and bacterial pneumonia agents were excluded in all patients by respiratory viral panel and respiratory and blood cultures. Patients were subsequently divided into laboratory-confirmed (RT-qPCR +) and clinically diagnosed (RT-qPCR -, thoracic CT, and clinical findings +) depending on their nasal swab specimen results. Blood specimens (10 ml) were collected from all patients immediately on hospitalization from a large vein in the antecubital region between 08.00 and 10.00 a.m. after 12-h fasting. Hemoglobin, platelet and lymphocyte counts, Mean Platelet Volume (MPV), activated Partial Thromboplastin Time (aPTT), International Normalized Ratio (INR), fibrinogen, D-dimer, creatinine, albumin, Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Lactate Dehydrogenase (LDH), C-Reactive Protein (CRP), and ferritin measurements were performed on the same day. Blood specimens obtained for SCUBE1 and sCD40 L measurement were immediately centrifuged for 10 min at 2500 x g for serum collection. Serum specimens were then stored at -80 C until the day of study.

SARS-CoV-2 RT-qPCR Test

RT-qPCR tests involving oropharyngeal and nasopharyngeal swab specimens from patients presenting to our hospital on suspicion of COVID-19 infection were performed at the Medical Microbiology Laboratory. The swab specimens were placed into tubes containing 2-3 ml vNAT™ buffer. Viral RNAs were extracted in vNAT™ buffer with no additional extraction procedure. Amplification of Open Reading Frame 1ab (ORF1ab) and nucleocapside protein (N) target genes was performed using appropriate SARS-CoV-2 Double Gene RT-qPCR kits (Bioeksen R&D Technologies Ltd., Istanbul, Turkey) in line with the manufacturer's instructions. Reaction mixtures were prepared to a total volume of 20 µl with the addition of 10 µl 2X Prime Script Mix, 5 µl CVD Di Oligo Mix, and 5 µl template nucleic acid. RT-qPCR tests were performed with reverse transcriptional reaction for 5 min at 52°C, pre-denaturation at 95°C for 10 sec, 40 cycle denaturation at 95°C for 1 sec and 40 cycle extension at 55°C for 30 sec, and fluorescence signal collection. Non-sigmoidal curves were defined as negative, while cycle threshold values (Cq) <38 were regarded as positive test results. Repeat specimens were requested for samples with Cq ≥ 38, and the RT-qPCR tests were repeated.

Measurement of sCD40L levels

Human sCD40L levels were measured using a Bioassay Technology Laboratory (Shanghai Korain Biotech Co. Ltd. Shanghai, China) commercial ELISA kit (catalogue no. E0251Hu, sensitivity 0.027 ng/ml, intra-assay variation coefficient (CV) <8%, inter-assay CV < 10%)

Measurement of Human SCUBE1 levels

Human SCUBE1 levels were measured using a Bioassay Technology Laboratory (Shanghai Korain Biotech Co. Ltd. Shanghai, China) commercial ELISA kit (catalogue no. E3142Hu, sensitivity 0.55ng/ml, intra-assay CV <8%, inter-assay CV < 10%).

Statistical Analysis

Compatibility with normal distribution was evaluated using the Kolmogorov-Smirnov test. The t test was applied in the comparison of normally distributed data, and the Mann Whitney-U test for non-normally distributed data. The chi-square test was employed in the evaluation of demographic variables, and Pearson correlation analysis for determining correlations. p values <0.05 were regarded as statistically significant.

Results

Demographic data and biochemical parameters

Comparison of the 24 clinically diagnosed (mean age: 52.08 ± 18.06) (11 F/13 M) and 27 laboratory-confirmed (mean age: 49.30 ± 15.74) (15 F/12 M) COVID-19 cases revealed no significant difference between the two groups in terms of age or sex. However, time from onset of COVID-19 symptoms was significantly greater in the clinically diagnosed patients (<0.001). While there was no difference between the two groups in terms of disease symptoms, history of contact with COVID-19 patients was significantly greater among laboratory-confirmed patients (<0.005) (Table 1).

In terms of biochemical and hematological parameters, CRP and fibrinogen levels and lymphocyte counts were significantly higher in the clinically diagnosed group (p 0.005, <0.05, and <0.05, respectively). No significant difference was observed in other biochemical and hematological parameters (Table 2).

SCUBE 1 and sCD40 L levels

SCUBE 1 levels were 23.26 (3.74-82.99) ng/mL in the clinically diagnosed group and 31.01 (12.26-242.10) ng/mL, significantly

Table 1: A comparison of the demographic parameters of the clinical diagnosed and laboratory-confirmed COVİD-19 pateients.

	Clinical diagnosed (n:24)	Laboratory confirmed (n:27)	P values
Age (year)	52.08 ± 18.06	49.30 ± 15.74	NS
Gender (F/M)	11/13	15/12	NS
SBP (mmHg)	12.59 ± 13.44	123.25 ± 20.37	NS
DBP (mmHg)	76.76 ± 9.51	77.04 ± 12.88	NS
Time of onset of symptoms	4.5 (1-20)	1 (0-12)	< 0.001
(Day)			
Loss of taste (%)	0	11	NS
Chills (%)	8	29	NS
Sore throat (%)	12	11	NS
Shortness of breath (%)	33	25	NS
Cough (%)	83	59	NS
Faever (%)	62	48	NS
Fatigue (%)	16	25	NS
Diarrhea (%)	8	7	NS
Suspicious contact (%)	8	48	< 0.005
Headache (%)	4	7	NS

Data are presented as arithmetic mean ± standard deviation, percent or median (min-max). Statistical significance was set at P<0.05.
Abbreviations: DBP; Diastolic Blood Pressure, SBP; Systolic Blood Pressure.

Table 2: A comparison of the biochemical and hematological parameters of the clinical diagnosed and laboratory-confirmed COVİD-19 pateients.

	PCR-group (n:24)	PCR + group (n: 27)	P values
Glucose (mg/dL)	119.50 (71-217)	112 (87-457)	NS
Creatinine (mg/dL)	1.41 (0.48-8.94)	0.86 (0.49-2.38)	NS
Albumin	4.24 ± 0.56	4.22 ± 0.73	NS
AST	24.90 (10-193)	27.90 (11-113)	NS
ALT	19.55 (3-83)	21 (9-55)	NS
LDH	256 (154-459)	229(141-977)	NS
CRP (mg/dL)	40.40 (7.54-223.70)	11.88 (0.78-164.32)	0.005
Ferritin	139.55 (14-1202)	147.80 (14-2736)	NS
Hemoglobin (g/dL)	13.15 ± 2.59	13.54 ± 2.12	NS
MPV	8.67 ± 0.98	8.99 ± 1.02	NS
Platelet (x10$^{3/}$ μL)	226.38 ± 78.57	193.33 ± 56.33	NS
Lenfosit	1.79 ± 1.16	1.26 ± 0.49	P<0.05
INR	1.10 ± 0.05	1.05 ± 0.09	NS
aPTT (sn)	24.61 ± 2.91	24.58 ± 2.88	NS
Fibrinogen (mg/dL)	436.59 ± 142.91	350.08 ± 134.99	P<0.05
D dimer (ng/mL)	0.80 (0.19-35.20)	0.56 (0-11.13)	NS
SCUBE 1	23.26(3.74-82.99)	31.01(12.26-242.10)	P<0.05
sCD40 L	1.07(0.26-4.17)	2.28(0.43-16.80)	0.05

Data are presented as arithmetic mean ± standard deviation, percent or median (min-max). Statistical significance was set at P<0.05.
Abbreviations: aPTT: activated partial thromboplastin time, AST: aspartate aminotransferase, ALT: alanine aminotransferase, CRP: C-reactive protein, INR: international normalized.

higher, in the laboratory-confirmed patients ($p < 0.05$). sCD40 L levels were 1.07 (0.26-4.17) ng/mL in the clinically diagnosed group, compared to 2.28 (0.43-16.80) ng/mL, also significantly higher, in the laboratory-confirmed group (p=0.005) (Table 2).

Parameters affecting SCUBE 1 and sCD40 L levels

Positive correlation was determined between SCUBE and sCD40 L levels in the entire patient group (p=0.000, r=0.979). SCUBE 1 levels were negatively correlated with ferritin and CRP (p< 0.05, r= - 0.322 and p< 0.05, r= - 0.351, respectively), but there was no correlation between sCD40 L and these parameters. No correlation was also determined between other biochemical and hematological parameters and SCUBE1 or sCD40 L.

Discussion

Levels of the platelet activation markers SCUBE1 and sCD40 L in this 51-case study were significantly higher in the laboratory-confirmed COVID-19 patients than in the clinically diagnosed patients. However, CRP and fibrinogen levels and lymphocyte counts were significantly higher in the clinically diagnosed COVID-19 patients.

COVID-19 disease is currently diagnosed by means of RT-qPCR, antibody screening in circulation using ELISA tests, and/or chest CT. Various studies have described RT-qPCR as the fastest and most reliable diagnostic tool, although it may be insufficient for diagnosis in some patients. RT-qPCR exhibits a particularly high diagnostic rate in samples collected 2-4 days after the onset of symptoms [18-20]. However, RT-qPCR may be inadequate for diagnosis. Factors affecting RT-qPCR positivity include inaccurate specimen collection or errors during transportation to the laboratory environment, disease prevalence rates in society, and how soon specimens are obtained after the onset of symptoms [21]. Diagnosis was slightly delayed in RT-qPCR negative patients, particularly in the early stages of the pandemic, which has now lasted for approximately a year. Increased clinical observations and scientific data have meant that COVID-19 can now be diagnosed through clinical symptoms, chest CT findings, and various laboratory abnormalities. However, data are still lacking for clinical and laboratory differences in laboratory-confirmed and clinically diagnosed patients and for mortality and morbidity expectations. There was no significant difference in demographic data between the laboratory-confirmed and clinically diagnosed patients in the present study. Symptoms were also similar in the two groups, but time from onset of symptoms was longer in the clinically diagnosed patients. In other words, these patients presented to hospital was later. Additionally, there was a greater history of contact with COVID-19 patients in the laboratory-confirmed group. Patients with a history of contact appear to have suspected the disease more quickly and therefore to have presented to a health institution earlier. Levels of the inflammatory markers CRP and fibrinogen were higher in the clinically diagnosed patients. The fact that these patients presented to hospital later after the onset of symptoms than the laboratory-confirmed patients may have resulted in higher inflammatory marker levels in these patients. However, the platelet activation markers SCUBE 1 and sCD40 L were significantly higher in the laboratory-confirmed patients.

The SARS-CoV-2 virus leads to various hematological abnormalities, as in other viral infections. These hematological abnormalities are used as an indicator in disease suspicion and

follow-up. Commonly seen hematological abnormalities include lymphopenia, D-dimer and fibrinogen elevation, neutrophilia, mild thrombocytopenia, or more rarely thrombocytosis [22]. Studies have shown that D-dimer elevation, lymphopenia, and thrombocytopenia indicate poor prognosis [2]. In contrast to classic DIC caused by bacterial infections, the SARS-COV-2 virus causes coagulation disorder, resulting in mild prolongation of PT/aPTT, mild thrombocytopenia, rarely hypofibrinogenemia, and rarely an increase in parameters supporting increased fibronolysis. COVID-19 related coagulopathy is therefore still the subject of research [11]. The purpose of the present study was also to investigate whether coagulation parameters would differ between clinically diagnosed and laboratory-confirmed COVID-19 patients and to compare the levels of the platelet activation markers SCUBE 1 and sCD40 L.

SCUBE1 is a cell surface protein and member of the SCUBE gene family [15]. It has been shown to be stored in rapidly proliferating tissues during embryogenesis, the endothelium, and finally in platelet alpha granules [15,16]. SCUBE1 protein is predominantly expressed by platelets and can be converted proteolytically into small active fragments and released. These active fragments are associated with thrombus formation. Molecular and biochemical studies have also shown that SCUBE 1 is an adhesive molecule involved in platelet-matrix interaction and ristocetin-induced platelet agglutination [17]. Two previous studies performed in our clinic also showed significantly higher SCUBE 1 in a hypertension and hemodialysis patient group disposed to clotting than in healthy individuals [23,24]. Menteşe et al. also determined significant SCUBE 1 elevation in patients with Crimean-Congo hemorrhagic fever, a viral infection with a fatal course 25. CD40 is a 50 kD transmembrane protein and member of the tumor necrosis factor superfamily. CD40L was originally identified on the CD4 T cell, although it has also recently been found on activated platelets. Several studies have shown that it is an important platelet activation marker [12,14]. To the best of our knowledge, the present study is the first to compare levels of the platelet activation markers SCUBE1 and sCD40L in clinically diagnosed and laboratory-confirmed COVID-19 patients, and both were found to be significantly higher in the laboratory-confirmed patients.

In conclusion, in the light of the current data, varying levels of coagulation are observed during the course of the disease in COVID-19 patients. Understanding the pathophysiology of the coagulation process and identifying the target molecules will be useful in terms of early initiation of anticoagulant or antiplatelet therapies. The findings of the present study show that platelet activation markers are significantly higher in laboratory-confirmed COVID-19 patients, who also present to hospital earlier. Higher levels of platelet activation markers, the main and triggering cells of the coagulation process, in laboratory-confirmed COVID-19 patients may be related to these patients presenting to hospital earlier. In the light of our findings, we think that early initiation of antiplatelet therapies in these patients will play an important role in the control of the coagulation process. We also think that our findings now need to be confirmed by further prospective studies with larger patient numbers.

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Conflict of Interest

We report no conflict of interest.

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