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Elevated plasma levels of soluble C-type lectin-like receptor 2 (CLEC2) in patients with thrombotic microangiopathy

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ABSTRACT

Background: Thrombotic microangiopathy (TMA) is caused by activated platelets. The plasma C-type lectin-like receptor 2 (CLEC2) levels in 58 patients with TMA were examined and compared with those in healthy volunteers and other diseases.

Materials and methods: The plasma levels of soluble platelet surface glycoprotein VI (GPVI) and CLEC2 were measured in patients with TMA.

Results: Plasma CLEC2 levels in patients with DIC and TMA were significantly higher ($p < 0.001$) than those in thrombocytopenic patients with other hematological diseases, but no significant differences in the plasma CLEC2 levels were observed among patients with thrombotic thrombocytopenic purpura, hemolytic uremic syndrome (HUS), atypical HUS and other TMA. The plasma CLEC2 levels after the remission were significantly lower than those before treatment ($p < 0.001$). The plasma CLEC2 levels were poorly correlated with the levels of soluble GPVI in the plasma of patients with TMA. The plasma CLEC2 levels were not significantly differ between survivor and non-survivor in TMA patients, but were significantly higher in non-survivor in overall population ($p < 0.001$).

Conclusion: The measurement of the plasma CLEC2 level is considered to be important for the diagnosis and evaluation of TMA.

1. Introduction

Thrombotic microangiopathies (TMAs) [1,2] such as thrombotic thrombocytopenic purpura (TTP) [3,4], hemolytic uremic syndrome (HUS) [5], atypical HUS (aHUS) [6] and other secondary TMA [1] are defined by the association of acute mechanical hemolytic anemia, thrombocytopenia, and visceral ischemic manifestations related to the formation of platelet thrombi in the microcirculation. TTP is caused by a markedly decreased ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13) level and unusually large multimers of Von Willebrand factor (ULM-VWF) [7]. HUS with diarrhea and melena consists of Shiga toxin-producing *Escherichia coli*-HUS (STEC-HUS) [5] and aHUS caused by genetic abnormalities affecting the complement regulatory system [6].

The plasma levels of soluble thrombomodulin [8], P-selectin [9], VWF and VWF propeptide (VWFpp) [10] were reported to be elevated in patients with TMA as markers of activated or damaged vascular endothelial cells. Following platelet activation, platelet factor 4 (PF4), β-thromboglobulin (β-TG) and P-selectine were previously used; however, these markers showed low specificity for thrombosis due to platelet activation. Elevated plasma levels of platelet surface glycoprotein VI (GPVI) were reported in patients with TMA [11], postoperative patients [12,13] and those with acute coronary syndrome [14,15]. Furthermore, C-type lectin-like receptor 2 (CLEC2) has been identified as a platelet receptor for a platelet-activating snake venom, rhodocytin [16]. CLEC-2 belongs to the C-type lectin superfamily and elicits activation signals in conjunction with the Src family kinases and Syk [16], suggesting that the plasma CLEC2 levels might be high in patients with suspected platelet activation. In soluble CLEC2, 25 kDa and 32/40 kDa bands were detected by western blotting; the 25 kDa band was

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considered to be cleaved by protease and the 32/40 bands were considered to be intact CLEC2 [18].

This study examined the activation of platelets by measuring the plasma CLEC2 levels in 58 patients with TMA, 40 healthy volunteers (HVs), 32 patients without thrombosis, 13 patients with old thrombosis, 14 patients with thrombophilia and 11 patients with disseminated intravascular coagulation (DIC).

2. Materials and methods

The plasma CLEC2 levels were measured in 58 patients with TMA (female, $n = 30$; male, $n = 28$, median age: 55.0 years [25th–75th percentile: 34.0–69.0 years]) who were measured at Mie University Hospital from April 1, 1990 to March 31, 2016 and were compared to those in 40 HVs (female, $n = 18$; male, $n = 22$, median age: 22.0 years [22.0–26.0 years]) 32 patients without thrombosis (female, n = 18; male, $n = 14$, median age: 63.5 years [51.0—68.0 years]) 14 patients with thrombophilia (female, $n = 14$; median age: 43.0 years [31.0–56.0 years]), 13 patients with old thrombosis (female, $n = 12$; male, n = 1, median age: 53.0 years [34.8–58.0 years]) and 11 patients with DIC (female, $n = 5$; male, $n = 6$, median age: 45.0 years [36.0–65.0 years]). The patients without thrombosis included 8 patients with connective tissue disease, 7 with solid cancer and 17 with hematopoietic disease. Five patients were positive for antiphospholipid antibody, 5 patients showed congenital antithrombin deficiency, 3 patients with congenital protein C deficiency and one patient with congenital protein S deficiency with thrombophilia. The patients with old thrombosis had been treated with anticoagulants and had not shown symptoms or laboratory findings associated with thrombosis for more than one year. TMA was diagnosed according to the diagnostic criteria of TMA; thrombocytopenia ($\leqq 10 \times 10^4/\mu l$), hemolytic anemia (hemoglobin $\leqq$10.0 g/dl) due to microangiopathy (the presence of fragmented red cells, elevated total bilirubin and LDH) and organ failure including neurological or renal dysfunction [1]. The TMA patients who had an $\leqq$10% ADAMTS 13 activity, were diagnosed with TTP and TMA patients who had an STEC infection, were diagnosed with STEC-HUS, and TMA patients who had a genetic abnormality affecting the complement system, were diagnosed with aHUS. DIC was diagnosed using the International Society of Thrombosis Haemostasis overt-DIC diagnostic criteria [17].

The study protocol was approved by the Human Ethics Review committees of Mie University School of Medicine, and signed informed consent was obtained from each patient.

2.1. Measurement of the CLEC2, GPVI and ADAMTS13 levels

The plasma CLEC2 and GPVI levels were quantified by a sandwich ELISA using previously described methods [18,19]. The ADAMTS13 level was measured using FRETS-VWF73, which was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) according to the method described by Kokame et al. [20,21].

2.2. Statistical analysis

The data are expressed as the median (25th to 75th percentile). Differences between groups were examined for significance using the Mann-Whitney *U* test. *P*-values of $\leqq$0.05 were considered to indicate statistical significance. The correlation between the CLEC2 and sGPVI was examined using Spearman's rank correlation coefficient.

All statistical analyses were performed using the Stat flex software program (version 6. Artec Co Ltd., Osaka, Japan).

3. Results

The plasma CLEC2 levels in patients with DIC (0.128 ng/ml; 0.019–0.713 ng/ml) and TMA (0.119 ng/ml; 0.030–0.270 ng/ml) were significantly higher than in HV (0.001 ng/ml; 0.001–0.015 ng/ml), patients with old thrombosis (0.001 ng/ml; 0.001–0.007 ng/ml), thrombophilia (0.001 ng/ml; 0.001–0.014 ng/ml) and patients without thrombosis (0.002 ng/ml; 0.001–0.017 ng/ml, Fig. 1). The plasma CLEC2 levels of patients with TMA and those with DIC did not differ to a statistically significant extent. In patients with TMA, there were no significant differences in the plasma CLEC2 levels of patients with TTP (0.077 ng/ml; 0.016–0.162 ng/ml), aHUS (0.072 ng/ml; 0.031–0.262 ng/ml), STEC-HUS (0.277 ng/ml; 0.012–0.716 ng/ml) and other TMA (0.181 ng/ml; 0.065–0.584 ng/ml, Fig. 2), however those levels were significantly higher than the plasma CLEC2 levels of HVs, patients with thrombophilia or old thrombosis, and patients without thrombosis. The ADAMTS13 levels in the patients with TTP (0%: 0–2.81%) were significantly lower than those in all other groups and the plasma GPVI levels among the TTP, aHUS, STEC-HUS, other-TMA

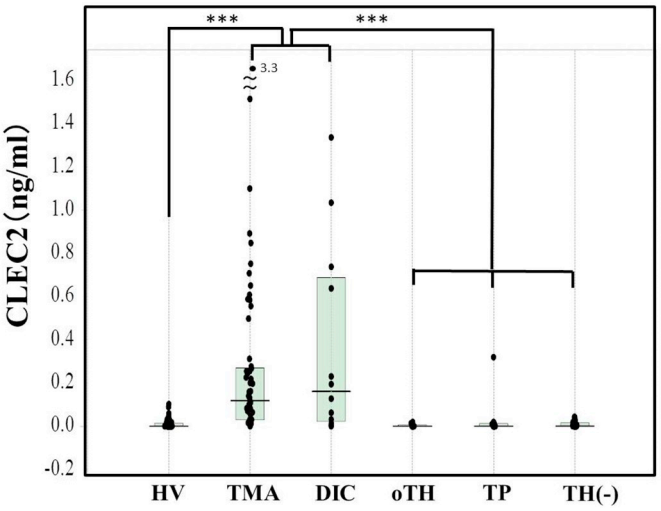

Fig. 1. Plasma levels of soluble CLEC2 in healthy volunteers and patients with various diseases.
HV, healthy volunteer; TMA, thrombotic microangiopathy; DIC, disseminated intravascular coagulation; oTH, old thrombosis; TP, thrombophilia; CLEC2, C-type lectin-like receptor 2; ***, $p < 0.001$.

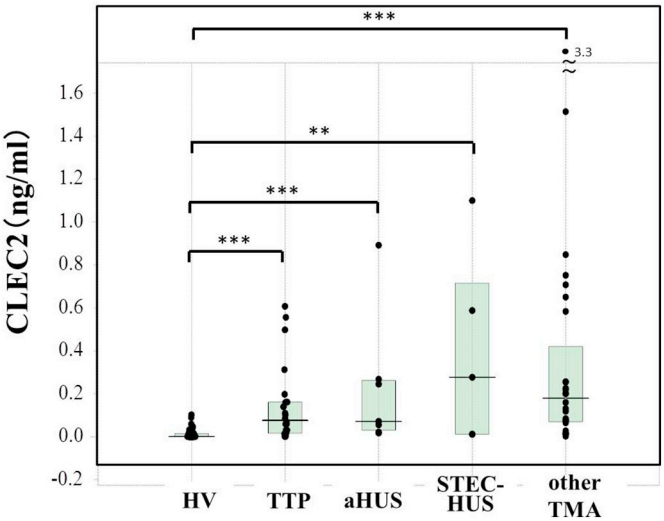

Fig. 2. Plasma levels of soluble CLEC2 in healthy volunteers, TTP, aHUS, STEC-HUS and other TMAs.
TTP, thrombotic thrombocytopenic purpura; aHUS, atypical hemolytic uremic syndrome; STE-HUS, Shiga toxin-producing *Escherichia coli*–HUS; TMA, thrombotic microangiopathy; CLEC2, C-type lectin-like receptor 2; ***, $p < 0.001$; **, $p < 0.01$.

Table 1
Patients with TMA or DIC.

		TTP	aHUS	STEC-HUS	Other-TMA	DIC
Number		23	7	3	25	11
Age		61.0 (48.8–74.8)	26.0 (12.3–35.3)	15.0 (1.0–16.0)	55.0 (36.3–62.8)	45.0 (36.0–65.0)
Female:Male		12:11	3:4	2:1	13:12	5:6
CLEC2 (ng/ml)	Median 25th–75th percentile	0.077 (0.016–0.162)	0.072 (0.031–0.262)	0.277* (0.012–0.716)	0.181 (0.065–0.584)	0.128 (0.019–0.713)
ADAMTS13 (%)	Median 25th–75th percentile	0 (0–2.81)	67.5 (39.9–80.0)	62.5* (57.5–76.3)	37.6 (16.3–57.5)	38.1 (22.5–68.8)
GPVI (ng/ml)	Median 25th–75th percentile	36.5 (27.5–48.1)	53.9 (43.1–57.5)	33.2* (12.1–43.9)	55.7 (28.4–67.0)	34.0 (30.0–53.9)
Death (mortality)		5 (21.7%)	0 (0%)	0 (0%)	7 (28.0%)	3 (27.2%)

Data are shown as the median (25th–75th percentile), except for STEC-HUS* as the median (range).

Table 2
CLEC2 and GPVI levels in patients with thrombocytopenia due to TMA, DIC or hematological diseases.

	TMA	DIC	Hematological diseases
CLEC2 (ng/ml)	0.093 (0.017–0.256)***	0.162 (0.023–0.688)***	0.0001 (0.0001–0.0098)
GPVI (ng/ml)	43.36 (27.30–141.9)***	33.9 (24.38–50.80)**	14.2 (2.55–23.65)

Data are shown as the median (25th–75th percentile).
*** $p < 0.001$.
** $p < 0.01$ in comparison with other hematological diseases with thrombocytopenia.

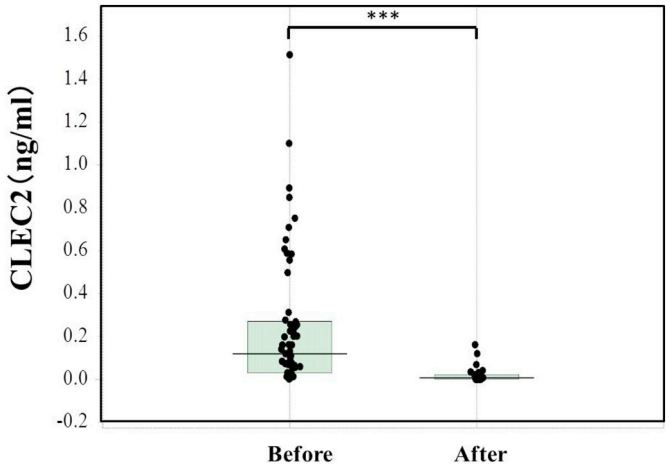

Fig. 3. Plasma levels of soluble CLEC2 in patients with TMA before and after the remission.
CLEC2, C-type lectin-like receptor 2; ***, p < 0.001.

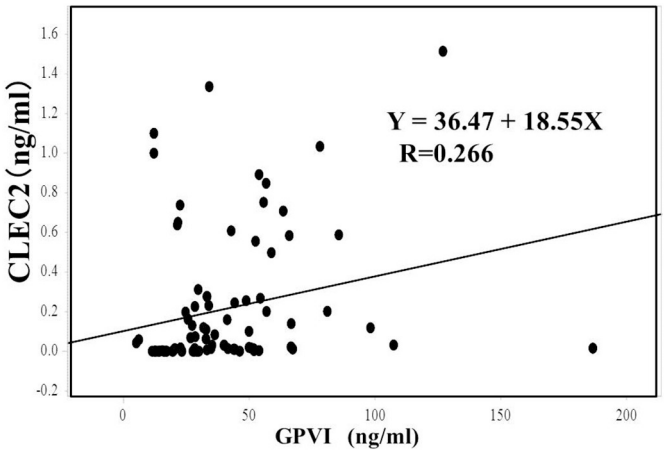

Fig. 4. Correlation between the plasma levels of soluble CLEC2 and GPVI in patients with TMA.
CLEC2, C-type lectin-like receptor 2, GPVI, platelet surface glycoprotein VI; TMA, thrombotic microangiopathy.

and DIC groups did not differ to a statistically significant extent (Table 1). However, the plasma CLEC2 and GPVI levels in patients with TMA were significantly higher than those in patients with other hematological diseases with thrombocytopenia, such as idiopathic thrombocytopenic purpura, aplastic anemia and patients after chemotherapy (Table 2). After treatment of TMA with methods such as plasma exchange, plasma infusion, antithrombin and recombinant thrombomodulin, and treatment of the underlying diseases, 27 plasma samples from TMA patients who had complete remission were obtained. The plasma CLEC2 levels after treatment (0.007 ng/ml; 0.001–0.022 ng/ml) were significantly lower than those before treatment (Fig. 3). Plasma CLEC2 levels were poorly correlated with the plasma GPVI levels in patients with TMA ($Y = 36.47 + 18.55X$, $R = 0.266$, Fig. 4). The plasma CLEC2 and GPVI levels were not well correlated with the platelet number, the hemoglobin, LDH and creatinine levels (Table 3).

The mortality was 21.7% in TTP, 0% in aHUS and STEC-HUS, 28.0% in other-TMA and 27.2% in DIC (Table 1). Although the plasma CLEC2 levels of survivors (0.093 ng/ml; 0.028–0.255 ng/ml) and non-survivors (0.226 ng/ml; 0.054–0.603 ng/ml) did not differ to a statistically significant extent in TMA and DIC, the plasma CLEC2 levels in non-survivors were significantly higher than those in survivors in the overall population (Fig. 5).

Table 3
The relationship between CLEC2 and platelet number, hemoglobin, LDH and creatinine in patients with TMA.

	CLEC2	GPVI
Platelet number	$r = -0.088$ ($p = 0.606$)	$r = 0.128$ ($p = 0.486$)
hemoglobin	$r = 0.518$ ($p < 0.001$)	$r = 0.247$ ($p = 0.173$)
LDH	$r = 0.213$ ($p = 0.206$)	$r = 0.368$ ($p = 0.05$)
Creatinine	$r = -0.093$ ($p = 0.23449$)	$r = 0.361$ ($p < 0.05$)

4. Discussion

Both TMA and DIC have a disseminated microvascular thrombosis including hypercoagulable state, increased platelet activations and

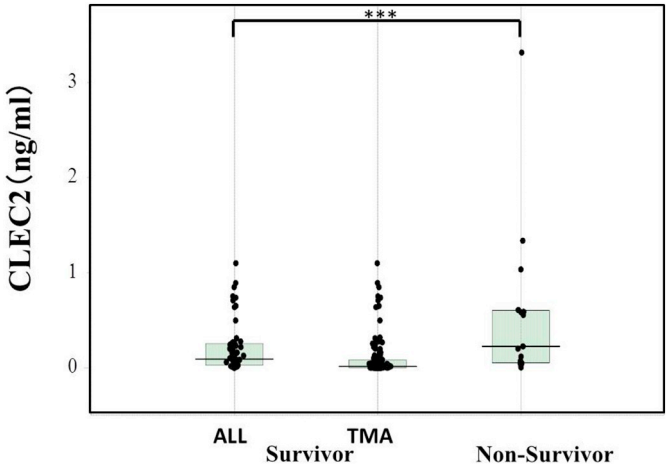

Fig. 5. The plasma levels of soluble CLEC2 in survivors and non-survivors. NS, not significant, CLEC2, C-type lectin-like receptor 2, ALL; all patients; TMA, thrombotic microangiopathy patients.

injury of vascular endothelial cells [1,2]. In routine assays, elevated levels of soluble fibrin and D-dimer show the activation of coagulation system [22], and elevated levels of TM and VWF show the activation or injury of vascular endothelial cells [23]. ADAMTS13 is useful diagnostic marker for TTP [24]. Although the activation of platelets is difficult to examine by routine tests such as β-thromboglobin (β-TG) and platelet factor 4 (PF4), which reflect platelet activation but require careful laboratory work and a low temperature centrifuge. Thus, the CLEC2 and sGPVI assays are more stable, easier to use and faster in comparison to theβ-TG and PF4 tests. When CLEC2 and sGPVI are present in the plasma, they are considered to have been released from activated platelets.

The plasma CLEC2 levels were significantly high in patients with DIC and TMA and were significantly decreased after treatment, suggesting that the platelets in patients with TMA or DIC are markedly activated. Elevated sGPVI levels were previously reported in patients with TMA and patients with DIC [11]. As plasma CLEC2 levels are not increased in HVs or thrombophilia, old thrombosis or without thrombosis, these levels considered to be increased in acute, large and disseminated thrombosis. In the diagnosis of thrombocytopenia, the plasma CLEC2 levels in patients with TMA were significantly higher than those in patients with idiopathic thrombocytopenic purpura, aplastic anemia or post-chemotherapy, suggesting that plasma CLEC2 is useful for the differential diagnosis of thrombocytopenia. Plasma CLEC2 levels were poorly correlated with the plasma GPVI levels in patients with TMA, which were released from activated platelets [11–13]. Plasma CLEC2 levels were not increased in HVs or the patients without thrombosis but the plasma GPVI levels were slightly increased in these groups. As the distribution of plasma CLEC2 levels was wider in comparison to the plasma GPVI levels, plasma CLEC2 may be more useful for laboratory tests to measure platelet activation. Therefore, the mechanisms underlying the release from activated platelets might be different. The release of GPVI, which is collagen receptor, is cleaved by protease and the release of CLEC2 which is receptor for rhodocytin, may be caused to be cleaved by protease or to be increased microparticles of destroyed platelets [16].

Although TMA was classified as TTP, STEC-HUS, aHUS and other type of TMA in accordance with the causes, there were no significant differences in the plasma CLEC2 levels among TTP, STEC-HUS, aHUS and other type of TMA, indicating that the CLEC2 level is not useful for differential diagnosis of TMA. There were no significant differences in the plasma CLEC2 levels of survivors and non-survivors indicating that plasma CLEC2 levels might not be a predictor of the outcome; rather, they might reflect the efficacy of treatment. As the study population was relatively low, the relationship between the plasma CLEC2 levels and the outcome should be further examined. Elevated plasma sGPVI levels have been reported in patients with acute coronary syndrome and acute ischemic stroke [14,15], and after surgery [12,13], suggesting that plasma CLEC2 levels should be examined in patients with ischemic diseases or patients who have undergone surgery.

Disclosure of conflicts of interests

The sGPVI and CLEC2 ELISA systems were provided by Mochida Pharmaceutical Co. LTD. and LSI Medicine.

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