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ORIGINAL ARTICLE

Plasma levels of complement activation fragments C3b and sC5b-9 significantly increased in patients with thrombotic microangiopathy after allogeneic stem cell transplantation

Jiaqian Qi[1,2,3] · Jie Wang[1,2,3] · Jia Chen[1,2,3] · Jian Su[1,2,3] · Yaqiong Tang[1,2,3] ·
Xiaojin Wu[1,2,3] · Xiao Ma[1,2,3] · Feng Chen[1,2,3] · Changgeng Ruan[1,2,3] · X. Long Zheng[4] ·
Depei Wu[1,2,3] · Yue Han[1,2,3]

Abstract Transplantation-associated thrombotic microangiopathy (TA-TMA) is an uncommon but severe complication in patients undergoing allogeneic stem cell transplantation (allo-SCT). However, the mechanism is unclear. From 2011 to 2014, 20 patients with TA-TMA, 20 patients without, and 54 patients with various other complications, including veno occlusive disease (VOD), graft-versus-host disease (GVHD), and infection, were recruited in the study. Plasma vWF antigen (vWFAg), vWF activity (vWFAc), and *ADAMTS13* activity were determined in these patients by ELISAs and FRETS-vWF73 assay, respectively. Plasma C3b, sC5b-9, and CH50 were also determined by ELISAs. Plasma levels of C3b were significantly increased in patients with either TA-TMA ($p < 0.0001$) or GVHD ($p < 0.01$). Plasma sC5b-9 and CH50 levels in patients with TA-TMA were also significantly increased ($p < 0.001$). Plasma *ADAMTS13* activity was lower in patients with VOD, but normal with other complications. Both plasma vWFAg and vWFAc levels were not elevated in patients with TA-TMA or VOD compared with those of other groups. Complement activation likely via an alternative pathway (increased C3b, sC5b-9, and CH50) may play a role in the pathogenesis of TA-TMA. *ADAMTS13* activity is reduced in VOD, but the *ADAMTS13*/vWF axis appears to be unaffected in patients with TA-TMA.

Introduction

Transplantation-associated thrombotic microangiopathy (TA-TMA) is a potentially fatal complication following allogeneic stem cell transplantation (allo-SCT) [1, 2]. While it was recognized as a complication after allo-SCT in the early 1980s, its pathogenesis remains unclear [3–5]. Studies suggest that multiple factors resulting in endothelial cell injury, specifically, the abnormity of the complement system contribute to the pathogenesis [6, 7]. Complement can be activated via a classical, a lectin, and an alternative pathway, leading to the deposition of complement component C5b-9 complexes on endothelial cells and resulting in cell injury [8–10]. Jodele et al. observed complement regulatory defects in six patients developed TA-TMA after SCT (including 3 auto-SCT and 3 allo-SCT) [2]. These patients exhibited a high prevalence of a heterozygous CFHR3-CFHR1 deletion (83%) compared to that in the donor population (33%) [11]. Three of the six patients with TA-TMA after allo-SCT also demonstrated the presence of factor H autoantibodies [2]. Another study also showed that allo-SCT recipients with multiple complement gene variants (≥ 3) were at high risk for development of TA-TMA [12]. These results implied that increased

Jiaqian Qi, Jie Wang, and Jia Chen contributed equally

✉ Depei Wu
drwudepei@163.com

✉ Yue Han
hanyue@suda.edu.cn

1 The First Affiliated Hospital of Soochow University, Jiangsu Institute of Haematology, Suzhou, China

2 Soochow University, Collaborative Innovation Centre of Haematology, Suzhou, China

3 Key Laboratory of Thrombosis and Haemostasis of Ministry of Health, Suzhou, China

4 Divsion of Laboratory Medicine, Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL 35243, USA

numbers of complement gene variants predispose to TA-TMA, which contributes to the transplant-related mortality [12]. Arteriolar C4d deposition was found in the kidney disease following SCT [13]. Recently, an anti-C5 monoclonal antibody, eculizumab, has been used to treat TA-TMA with success [14–16]. Six children with severe TA-TMA were treated with eculizumab and four achieved remission over time [15]. Another study showed that eculizumab treatment in 12 TA-TMA patients led to hematological response and increased overall survival from 33 to 50% [14]. Thus, activation of the complement system may play a critical role in pathogenesis of TA-TMA.

However, TA-TMA has a similar clinical manifestation to autoimmune thrombotic thrombocytopenic purpura (TTP) with severe *ADAMTS13* deficiency [1, 2]. Because of the difficulty in making diagnosis, the incidence of TA-TMA was reported to be from 0.5 to 76% and the mortality of 60–90% [2, 6, 17]. Although some TA-TMA patients respond to instant plasma exchange treatment, most survivals have suffered from long-term complications, including hypertension, chronic kidney disease, gastrointestinal or central nervous system disease, and pulmonary hypertension [6, 18], which dramatically impacts the quality of life. Moreover, TA-TMA is often undistinguished from other complications associated with allo-SCT, including GVHD, severe infection, and veno occlusive disease (VOD) [19]. While severe deficiency of *ADAMTS13* activity is diagnostic for autoimmune TTP [20], TA-TMA patients usually have normal to modestly reduced plasma *ADAMTS13* activity [21]. Therefore, assays for plasma *ADAMTS13* help differential diagnosis and guides for therapy, but we still could not ignore those TA-TMA patients with anti-*ADAMTS13* antibody.

In the present study, we determined the plasma levels of complement activation markers including C3b, sC5b-9, and CH50 and the levels of von Willebrand factor (vWF) antigen, collagen-binding activity, and *ADAMTS13* activity in 20 patients with TA-TMA following allo-SCT and compared the results to those of 74 other patients without and with various other complications following allo-SCT. These include 20 patients receiving allo-SCT but no complications, 14 with VOD, 20 with GVHD, and 20 with severe infection. Statistical analysis was performed to assess the contribution of various biomarkers in pathogenesis of TA-TMA.

Materials and methods

Patients

Twenty patients diagnosed as TA-TMA who received allo-SCT at the first affiliated hospital of Soochow University from June 2011 through June 2014 were enrolled in this study. TA-TMA was diagnosed according to the criteria proposed by

Cho et al. [22]. The criteria include the following: schistocytes $\geq$ 2 per high-power field in peripheral blood, increased lactate dehydrogenase (LDH), thrombocytopenia $< 50 \times 10^9$/L or a 50% decrease in platelet count, decreased hemoglobin, negative of Coombs test, decreased haptoglobin, and no coagulopathy.

Seventy-four other patients following allo-SCT were randomly selected as control subjects including 14 cases of VOD, 20 cases of severe infections, 20 cases of 3–4 grade GVHD, and 20 cases with no complications. All the patients provided written informed consent for the protocol, which was approved by our hospital's Ethics Committee. Patients in the control group were matched with TA-TMA group for gender, age, and disease characteristic. Peripheral blood samples were collected and anti-coagulated with EDTA. Samples were then centrifuged at 800g for 10 min at room temperature to collect the supernatants that were stored at $-$ 80 °C for further assessments.

Conditioning regimen and GVHD prophylaxis

Both myeloablative [23] and reduced-intensity conditioning (RIC) [24] were applied for allo-SCT in our center. Two major myeloablative regimens included: (1) modified Bu/Cy regimen: Me-CCNU, cytarabine, busulfan, and cyclophosphamide, (2) modified TBI/Cy regimen: Me-CCNU, fractional total body irradiation (TBI), and cytarabine. The RIC regimen was composed mainly of fludarabine, cytarabine, and busulfan. Seventy-one percent of the patients received the modified Bu/Cy regimen. The modified TBI/Cy regimen was employed for patients with lymphoid malignancies and extramedullary diseases, while the RIC regimen for patients of advanced age or poor performance.

GVHD prophylaxis consisted of cyclosporine A (CsA) and short-term methotrexate (MTX), along with mycophenolate mofetil as well as antithymocyte globulin for unrelated and haploidentical donor SCT.

Selection criteria for comparison analysis

In order to determine the value of parameters in the panel, we set four control groups from the whole cohort for comparison analysis: VOD group: patients diagnosed as VOD according to the Baltimore Criteria [25]; GVHD group: patients with grades 3–4 GVHD as confirmed pathologically and graded using the International Bone Marrow Transplant Research criteria [26]; infection group: Infectious complications were defined by clinical and laboratory parameters [27]; and non-complications group: patients without clinical and laboratory indications of post-SCT complications including relapse, TA-TMA, VOD, GVHD, infections, and rheumatic diseases. We also remove cases with the overlap between those complications above.

Assays for plasma C3b, sC5b-9, and CH50 levels

Plasma samples were collected at two time points: before conditioning (BF) and at the onset of complications (AF). Samples were taken at "event diagnosis" when diagnosis of TMA, VOD, and GVHD was made clinically. For patients of non-complications group, plasma of AF was collected at 100 days of post SCT for well state of patients. Plasma levels of *C3b*, *sC5b-9*, and *CH50* were determined by ELISA (Xitang, Shanghai, China) according to the manufacturer's instruction.

Assays for plasma vWF antigen and activity

Plasma vWF antigen was determined by latex immunoassay (LIA). Plasma vWF activity was determined by collagen-binding assay using rabbit-anti-human vWF antibody (DAKO, NY, USA) for detection and type III collagen from placenta (Thermo, NY, USA) for capturing.

Assay for plasma ADAMTS13 activity

Plasma *ADAMTS13* activity was determined by fluorescence resonance energy transfer (FRETS)-vWF73 according to the method described previously [28, 29].

Statistical analysis

Statistical analyses were performed with SPSS 22.0 (IBM, Chicago, IL) and GraphPad Prism7 (La Jolla, CA). Differences of panel parameters among the groups were analyzed by non-parametric Kruskal-Wallis test. *P* values less than 0.05 were considered statistically significant between the groups.

Results

Patients

There is a total of 94 patients with male to female ratio of 53 is to 41, and the mean age is 31.4 ± 13 years old. Twenty patients had no complications, 20 had infection, 20 had 3–4 grade GVHD, 14 had VOD, and 20 had TMA. The mortality rates were 55% (TMA), 42.9% (VOD), 40% (infection), 55% (3–4 grade GVHD), and 5% (non-complications). Among 20 patients with TA-TMA, all the patients have been found with decreased hemoglobin thrombocytopenia (platelet $< 50 \times 10^9$/L), schistocytes ≥ 2 per high-power field in peripheral blood, and increased lactate dehydrogenase (LDH) (18 patients > 800 U/L, 2 patients > 600 U/L). In addition, 10 patients got psychosomatic manifestations.

No obvious alterations of vWF/ADAMTS13 axis in TA-TMA

Plasma vWF antigen, vWF activity, and *ADAMTS13* activity were not different among various groups except for the reduced levels of plasma vWF activity in patients with GVHD prior to conditioning and the reduced levels of *ADAMTS13* activity in patients with VOD (Table 1 and Fig. 1). These results suggest that vWF and *ADAMTS13* activities were not altered in TA-TMA patients, but *ADAMTS13* may be important for the development of VOD after allo-SCT.

Plasma C3b, sC5b-9, and CH50 levels were significantly increased in patients with TA-TMA

When compared with those before conditioning or those with no complications following allo-SCT, plasma C3b, *sC5b-9*, and *CH50* levels were significantly increased in patients with TA-TMA ($p < 0.001$). We also found C3b level increased in patients with GVHD ($p < 0.01$). No statistically significant difference among all other groups was detected (all *p* values > 0.05) (Table 1 and Fig. 2). These results demonstrated that plasma C3b, *sC5b-9*, and *CH50* levels may be a specific biomarker for TA-TMA following allo-SCT and C3b may also be a biomarker of GVHD.

Discussion

TA-TMA is a potentially fatal syndrome with a similar clinical feature to atypical hemolytic uremic syndrome (aHUS) and TTP. The pathogenesis of TA-TMA is not fully understood. In the present study, we find that plasma levels of C3b, sC5b-9, and CH50 are significantly increased in patients with TA-TMA compared with those with other complications or no complications following allo-SCT.

Factor H autoantibodies or gene variations or mutations are present in some patients with TA-TMA after SCT [12, 30]. Our findings suggest a role of complement activation in pathogenesis of TA-TMA following allo-SCT. Therapeutic intervention with anti-complement monoclonal antibody may be helpful in reducing morbidity and mortality.

Prompt and accurate diagnosis of TA-TMA following allo-SCT remains to be a challenge [5, 31]. Two different definitions for TA-TMA are proposed by the Blood and Marrow Transplants Clinical Trials Network (CTN) and International Working Group (IWG) [32, 33]. These different diagnostic criteria poss a further challenge for assessing the sensitivity and specificity for a given diagnostic test. In our study, we adopted Cho's diagnostic criteria, which does not require the presence of renal or neurologic findings for diagnosis [22]. The mortality rate in our cohort with TA-TMA is nearly 55%, which is similar to what has been reported in the

Table 1 Plasma levels of ADAMTS13 activity, von Willebrand antigen, collagen-binding activity, complement activation fragment C3b, and sC5b-9 in patients with various complications after stem cell transplantation

Conditions	N	ADAMTS13 (%)		vWF Ag (%)		vWF Ac (%)	
		Mean	Median (95% CI)	Mean	Median (95% CI)	Mean	Median (95% CI)
TMA_BF	20	83 ± 56	71 (56–91)	145 ± 26	142.5 (130.7–155.4)	128 ± 67	115.2 (92.8–142.9)
TMA_AF	20	69 ± 43	55 (39–79)	156 ± 29	153.5 (139.5–168.9)	153 ± 108	118.4 (82.5–169.8)
VOD_BF	14	63 ± 42	51 (34–77)	163 ± 30	159.9 (142.3–179.8)	174 ± 96	150.8 (108.4–209.9)
VOD_AF	14	36 ± 27**	26 (15–44)**	164 ± 32	160.8 (142.1–181.9)	215 ± 98	193.2 (143.5–260.0)
Inf_BF	20	106 ± 55	86 (58–128)	157 ± 42	149.5 (126.8–176.2)	140 ± 124	93.6 (59.6–146.9)
Inf_AF	20	106 ± 43	95 (74–121)	166 ± 53	151.1 (117.2–194.7)	235 ± 106	208.2 (161.7–268.0)
GVHD_BF	20	73 ± 39	61 (44–84)	155 ± 30	152.1 (138.1–167.5)	92 ± 107	50.1 (65.6–85.5)*
GVHD_AF	20	81 ± 52	62 (41–93)	172 ± 24	170.7 (160.2–182.0)	171 ± 120	112.3 (65.6–192.4)
None_BF	20	88 ± 56	74 (56–98)	159 ± 23	157.7 (147.5–168.7)	148 ± 170	83.3 (48.0–144.8)
None_AF	20	104 ± 72	86 (65–114)	168 ± 20	167.0 (157.9–178.6)	169 ± 98	130.0 (86.3–195.7)

Conditions	C3b (ng/mL)		sC5b-9 (ng/mL)		CH50 (U/L)	
	Mean	Median (95% CI)	Mean	Median (95% CI)	Mean	Median (95% CI)
TMA_BF	166 ± 98	138 (102–188)	673 ± 150	656 (584–736)	61.8 ± 23.0	60.3 (41.1–77.5)
TMA_AF	455 ± 224***	394 (300–519)***	1058 ± 276***	1025 (907–1158)***	128.4 ± 37.3**	128.4 (113.5–142.3)**
VOD_BF	212 ± 96	193 (150–250)	454 ± 197	420 (333–529)	77.9 ± 31.24	71.0 (55.6–105.3)
VOD_AF	248 ± 112	227 (177–292)	673 ± 256	622 (484–799)	87.98 ± 44.0	99.5 (42.1–116.2)
Inf_BF	213 ± 161	154 (102–232)	616 ± 224	577 (483–689)	77.0 ± 30.5	79.2 (51.9–100.3)
Inf_AF	202 ± 125	153 (102–231)	723 ± 348	657 (536–806)	60.4 ± 35.4	53.0 (31.7–93.2)
GVHD_BF	200 ± 120	163 (116–227)	559 ± 185	521 (427–636)	63.5 ± 24.7	64.4 (46.4–71.7)
GVHD_AF	299 ± 143**	252 (182–348)**	550 ± 167	522 (442–615)	64.7 ± 31.5	58.0 (48.4–85.3)
None_BF	211 ± 146	173 (128–235)	558 ± 197	515 (415–638)	68.1 ± 33.9	67.5 (44.7–90.6)
None_AF	154 ± 88	130 (97–174)	640 ± 155	621 (550–700)	54.5 ± 34.3	44.8 (28.2–85.2)

BF and AF indicate before and after complication occurred, respectively

TMA thrombotic microangiopathy, *VOD* venous occlusive disease, *Inf* infection, *GVHD* graft-versus-host hyperreactivity disease, *None* no complication controls
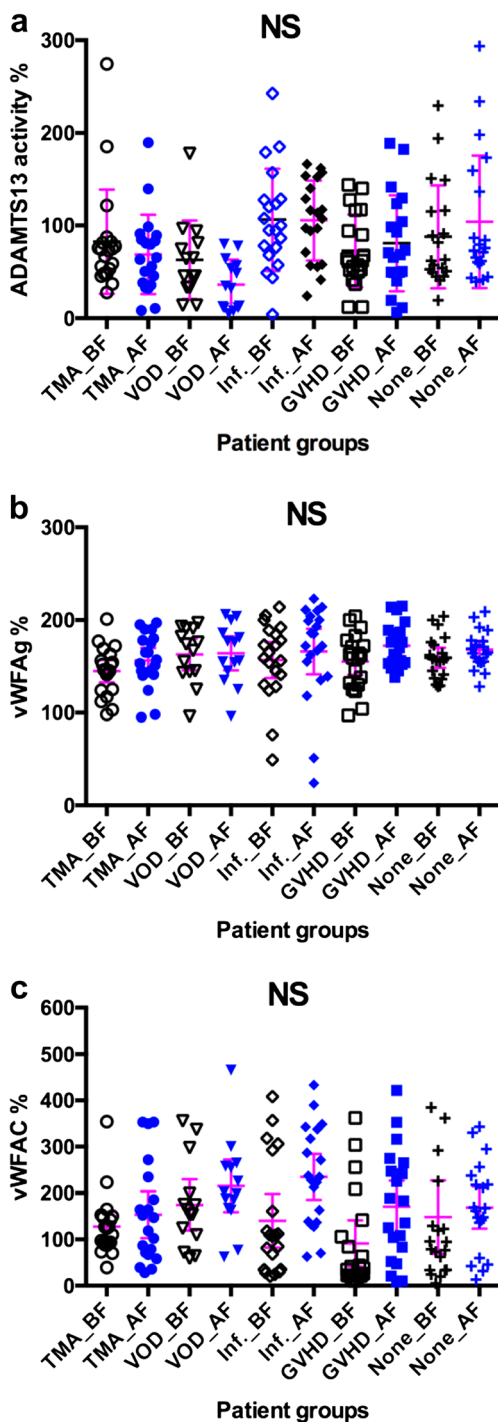
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Fig. 1 Plasma levels of vWF antigen, collagen-binding activity, and *ADAMTS13* activity in patients with TA-TMA and other complications following allo-SCT. Plasma levels of vWF antigen (**a**), collagen-binding activity (**b**), and *ADAMTS13* activity toward FRETS-vWF73 (**c**) were determined in patients with TMA (*n* = 20), VOD (*n* = 14), infection (*n* = 20), and GVHD (*n* = 20), as well as in those without complications (*n* = 20) before conditioning (BF) and during the complications (AF) or no complications (None_AF). *P* value > 0.05 is not statistically significant, *p* values < 0.05 (one star) and < 0.01 (two star) is considered to be statistically significant
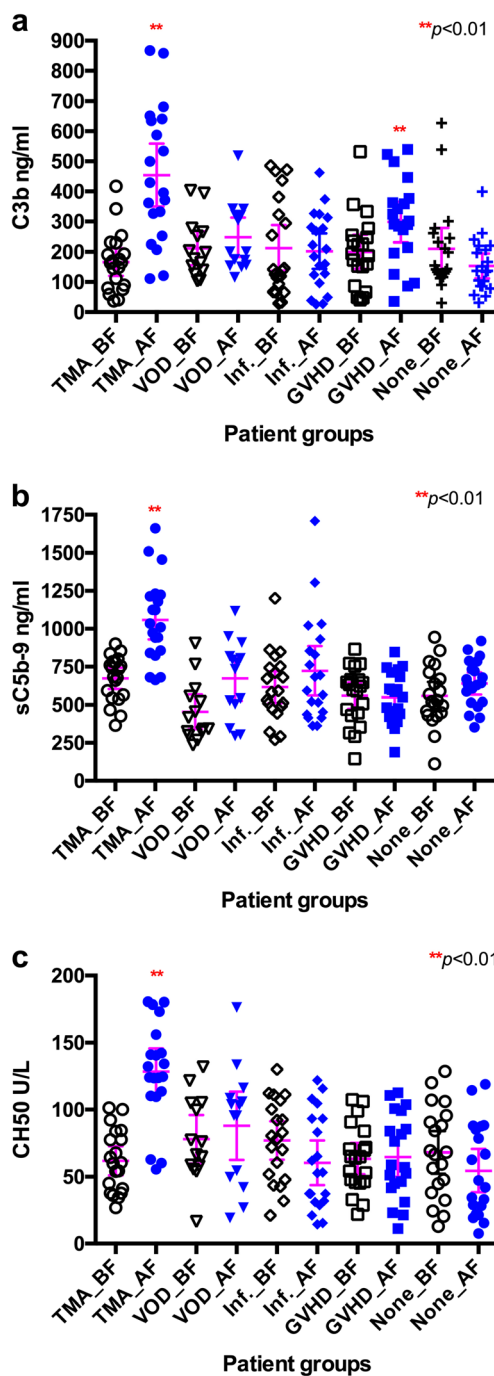
Fig. 2 Plasma levels of C3b, sC5b-9, and CH50 in patients with TA-TMA and other complications following allo-SCT. Plasma levels of C3b (**a**), sC5b-9 (**b**), and CH50 (**c**) before conditioning (BF) and during the complications (AF) including TMA (*n* = 20), VOD (*n* = 14), infection (*n* = 20), and GVHD (*n* = 20) or no complications during the follow-up as controls (None_AF, *n* = 20) in patients with TMA. *P* values < 0.05 and < 0.01 were considered to be statistically significant (**p* < 0.01: compared to None_AF group)

literature [1, 6, 32, 34–36]. The high mortality rate of TA-TMA patients calls for an urgent need of early biomarkers for diagnosis, thereby a specifically targeted therapy should be given for these patients.

In summary, plasma C3b levels are significantly increased in TA-TMA and GVHD patients, while sC5b-9 and CH50 increased specifically in TA-TMA. Plasma *ADAMTS13* activity is significantly reduced in patients with VOD, additionally, the elevated levels of plasma C3b, sC5b-9, and CH50 levels may be useful biomarkers for the early diagnosis of TA-TMA and GVHD. Our findings suggest the rationale for potential benefit of early use of anti-complement therapy in patients with probable TA-TMA. A multi-central trial will be critical to determine the efficacy of such an intervention to reduce the mortality rate associated with complications following allo-SCT.

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