bevacizumab/panitumumab: 1) 5-FU and folinic acid plus oxaliplatin (FOLFOX6), 2) capecitabine plus oxaliplatin (CapeOX), or 3) S-1 plus oxaliplatin (SOX). These treatments comprised adjuvant chemotherapy after radical surgery in 14 patients, chemotherapy for unresectable metastatic CRC in 11 patients, and neoadjuvant chemotherapy to treat advanced CRC in 5 patients. Six patients were excluded from this study for the following reasons: tumor progression (n = 3), self-discontinuation (n = 2), and adverse effects of chemotherapy (n = 1).

Ultimately, 23 CRC patients were analyzed in this study (Table 1): 12 patients who received adjuvant chemotherapy, 6 patients who received chemotherapy for unresectable metastatic CRC, and 5 patients who received neoadjuvant chemotherapy. Of these, 13 were treated with an oxaliplatin-based regimen only, 4 were treated with an oxaliplatin-based regimen plus panitumumab, and 6 were treated with an oxaliplatin-based regimen plus bevacizumab (Table 1). CALI was diagnosed based on the following 2 criteria: 1) serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), or total bilirubin (T-Bil) more than 2 times the upper limit of normal range at our hospital (ALT >70 IU/mL, AST >70 IU/mL, T-Bil 2.0 mg/dL) and 2) no other explanation for CALI.

This study was performed with the permission of the ethics committee of Nara Medical University and complied with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from each patient.

Blood sampling

Plasma samples were collected from CRC patients at 5 or 6 time points during chemotherapy every month, starting at baseline before chemotherapy (month 0) for 4 or 5 months after the initiation of chemotherapy. Chemotherapy was suspended when the patients underwent liver section for metastatic liver cancer. These samples were taken before the starting chemotherapy. Blood was collected in plastic tubes containing 1/10 volume of 3.8% sodium citrate. The plasma was separated by centrifugation at 3,000 g for 15 minutes at 4°C. Aliquots were stored at -80°C until use.

Assays of VWF antigen, activity, and ADAMTS13 activity

Plasma VWF:Ag levels were measured by sandwich ELISA using a rabbit anti-human VWF polyclonal antiserum (DAKO, Denmark) [16]. The value obtained from normal individuals (n = 20) in this assay was 102 ± 33% [16]. To determine the VWF activity, the collagen binding activity of plasma VWF (VWF:CB) was measured using a commercially available kit (VWF-CBA ELISA, PROGEN Biotechnik GmbH, Heiderberg, Germany) according to the manufactures's instructions. ADAMTS13:AC was determined using a commercially available chromogenic act-ELISA kit (Kainos Laboratories Inc., Japan) [17]. The value obtained for normal individuals (n = 55) in the act-ELISA was 99 ± 22%.17 The value of 100% was defined as the amount of VWF:Ag and ADAMTS13:AC in pooled normal human plasma (NP). To evaluate the both levels of VWF and ADAMTS13, the ratio of VWF:CB to ADAMTS13:AC was calculated in this study.

VWF multimer analysis

Multimer analysis of plasma VWF was essentially performed according to the method of Ruggeri and Zimmerman [18], with modifications as reported by Warren et al [19]. The lower gel consisted of 1% agarose (SeaKem® Gold Agarose, Lonza, Rockland, ME, USA) and 10% glycerol dissolved in 50 mmol/L phosphate buffer (pH 8.8) with 0.1% sodium dodecyl sulfate (SDS). The upper gel was prepared with 0.8% agarose (SeaKem® HGT Agarose, Cambrex, Rockland) dissolved in 370 mmol/L phosphate buffer (pH 6.8) with 0.1% SDS. The electrophoresis buffer consisted of 50 mmol/L Tris-glycine buffer (pH 8.3) containing 0.1% SDS. The

Table 1. Clinical characteristics, chemotherapy regimen, response of chemotherapy and spleen size ratio.

patient	age	sex	primary lesion	TNM stage	purpose	chemotherapy regimen	response #	spleen size ratio ##	CALI	SOS grade
1	71	M	rectum	IVA	neoadjuvant	FOLFOX6+Pan	NC	0.83		
2	54	F	rectum	IIC	adjuvant	SOX	no rec.**	0.92	yes	
3	46	F	rectum	IIIC	adjuvant	SOX	no rec.**	0.95		
4	58	M	sigmoid colon	IVA	neoadjuvant	FOLFOX6+Pan	PR	0.96	yes	Grade 2
5	56	F	rectum	IVA	UMCRC*	FOLFOX6	NC	1.07		
6	41	F	rectum	IIC	adjuvant	CapeOX	no rec.**	1.12	yes	
7	53	M	rectum	IVB	neoadjuvant	FOLFOX6+Pan	PR	1.19		Grade 2
8	56	M	rectum	IVA	adjuvant	CapeOX	no rec.**	1.2		
9	57	M	rectum	IVA	neoadjuvant	FOLFOX6+Pan	PR	1.52		
10	62	M	rectum	local rec.**	adjuvant	SOX	no rec.**	1.58		
11	48	M	rectum	IIIB	adjuvant	CapeOX	no rec.**	1.59	yes	Grade 0
12	73	F	transverse colon	peritoneal rec.	UMCRC*	CapeOX	NC	1.61		
13	50	F	descending colon	IVB	adjuvant	CapeOX	no rec.**	1.67		
14	62	F	sigmoid colon	IIC	adjuvant	CapeOX	no rec.**	1.68		
15	68	M	sigmoid colon	IVA	adjuvant	CapeOX	no rec.**	1.96		
16	69	M	rectum	IVA	adjuvant	CapeOX	no rec.**	2.3	yes	
17	59	M	rectum	IIIC	adjuvant	CapeOX	no rec.**	2.93	yes	
18	38	M	sigmoid colon	IVA	adjuvant	FOLFOX6+Bev	no rec.**	1.00		
19	57	M	sigmoid colon	IVB	UMCRC*	FOLFOX6+Bev	PR	1.02		
20	63	M	descending colon	IVB	UMCRC*	FOLFOX6+Bev	PR	1.07		
21	61	F	rectum	IVA	neoadjuvant	CapeOX+Bev	NC	1.09		Grade 1
22	67	F	transverse colon	IVB	UMCRC*	FOLOFOX6→FOLFOX6 +Bev	NC	1.17		
23	71	M	sigmoid colon	IVB	UMCRC*	FOLFOX6+Bev	PR	1.37		

tumor response was evaluated by Response Evaluation Criteria in Solid Tumors (RECIST). (response)
the spleen size ratio was the ratio of spleen size after chemotherapy to those before chemotherapy (spleen size ratio)
* unresectable metastatic colorectal cancer (UMCRC)
** recurrence (rec.)
Patients 18 through 23 were treated without bevacizumab.

experimental conditions including western blotting with luminographic detection were as previously described by Budde et al [20]. Multimers were classified as low molecular weight (corresponding to bands 1–5 in the VWFM analysis), intermediate molecular weight (bands 6–10), and H-VWFM (bands >10) [21]. High molecular weight bands that were not detected in NP were defined as UL-VWFMs.

Measurement of spleen size

Computed tomography (CT) to measure spleen size was performed on 2 occasions, before chemotherapy and 3–5 months after the start of chemotherapy. Spleen size was determined using the outline of the spleen on each axial CT image (5 mm section thickness). The sum of the area of the spleen in each section, taking into account slice thickness, was calculated by tracing the contour of the spleen using an electronic free curve tool provided by the software (Synapse®,

Tokyo, Japan). Splenomegaly was defined as a 50% increase over baseline in spleen size at 3–5 months after the start of chemotherapy [7]. The spleen size ratio was the ratio of spleen size after chemotherapy to those before chemotherapy. Therefore, spleen size ratio $\geq$ 1.5 was defined as having splenomegaly.

Immunohistochemistry in liver specimens

Serial sections were stained by hematoxylin and eosin (H. E.) to identify the basic constituents of thrombi. Immunohistochemistry staining was performed to clarify the distribution of platelets and fibrin. Formalin-fixed, paraffin-embedded tissues were cut into 5-μm sections, deparaffinized, and rehydrated in a graded series of ethanol. Antigen retrieval was done by heating tissue sections using Target Retrieval Solution at pH 6.0 (DAKO Japan, Kyoto, Japan). To block endogenous peroxidase activity, sections were immersed in a 0.3% solution of hydrogen peroxide in absolute methanol for 5 minutes at room temperature and washed 3 times with fresh PBS for 5 minutes each. Anti-IIb/IIIa (Affinity Biologicals, South Bend, Canada), anti-VWF (DAKO), anti-fibrin (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) antibodies were added to the sections, which were incubated overnight at 4°C. Sections were washed in PBS for 5 minutes thrice. We then used the ImmPRESS reagent kit, Mouse/HRP, or Rabbit/HRP (VECTOR) for VWF and fibrin, and anti-Sheep IgG (Jackson ImmunoResearch, West Grove, PA, USA) for IIb/IIIa according to the instructions of the manufacturer. Reaction products were visualized with 3,3′-diaminobenzidine (DAB) tetrahydrochloride. The sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and coverslipped.

Consecutive slices of non-tumor liver parenchyma were reviewed. The severity of sinusoidal congestion was graded from 0 to 3 as proposed by Rubbia-Brandt et al.:6 grade 0 = absent, grade 1 = mild (one-third of the lobule is affected), grade 2 = moderate (two-thirds of the lobule are affected), and grade 3 = severe (the entire lobule is affected).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test to compare the differences between groups, and Wilcoxon's signed rank test to compare the differences among any time points in each group. P<0.05 was considered statistically significant. Data are expressed as median (minimum-maximum). Statistical analysis was performed using GraphPad Prism software, version 6.01 (GraphPad Software, San Diego, CA, USA).

Results

Comparison between patients who received or did not receive bevacizumab

We classified patients into 2 groups: oxaliplatin-based chemotherapy with (n = 6) bevacizumab and without (n = 17) bevacizumab. As shown in Fig 1A, platelet counts decreased as the number of chemotherapy cycles increased among patients receiving bevacizumab (P = 0.002 at 3 months, P = 0.004 at 5 months) and not receiving bevacizumab (P = 0.031 at 3 months, P = 0.250 at 5 months). However, platelet counts at 5 months in patients not treated with bevacizumab decreased much less than in patients who received bevacizumab. In patients not treated with bevacizumab, plasma levels of VWF:Ag increased as the number of chemotherapy cycles increased (P<0.001 at 3 months, P = 0.027 at 5 months), but there was no change in patients treated with bevacizumab (P = 0.094 at 3 months, P = 0.156 at 5 months) (Fig 1B). Plasma levels of ADAMTS13:AC were unchanged in both groups (Fig 1C). Serum AST levels increased with the number of chemotherapy cycles in patients not treated with bevacizumab
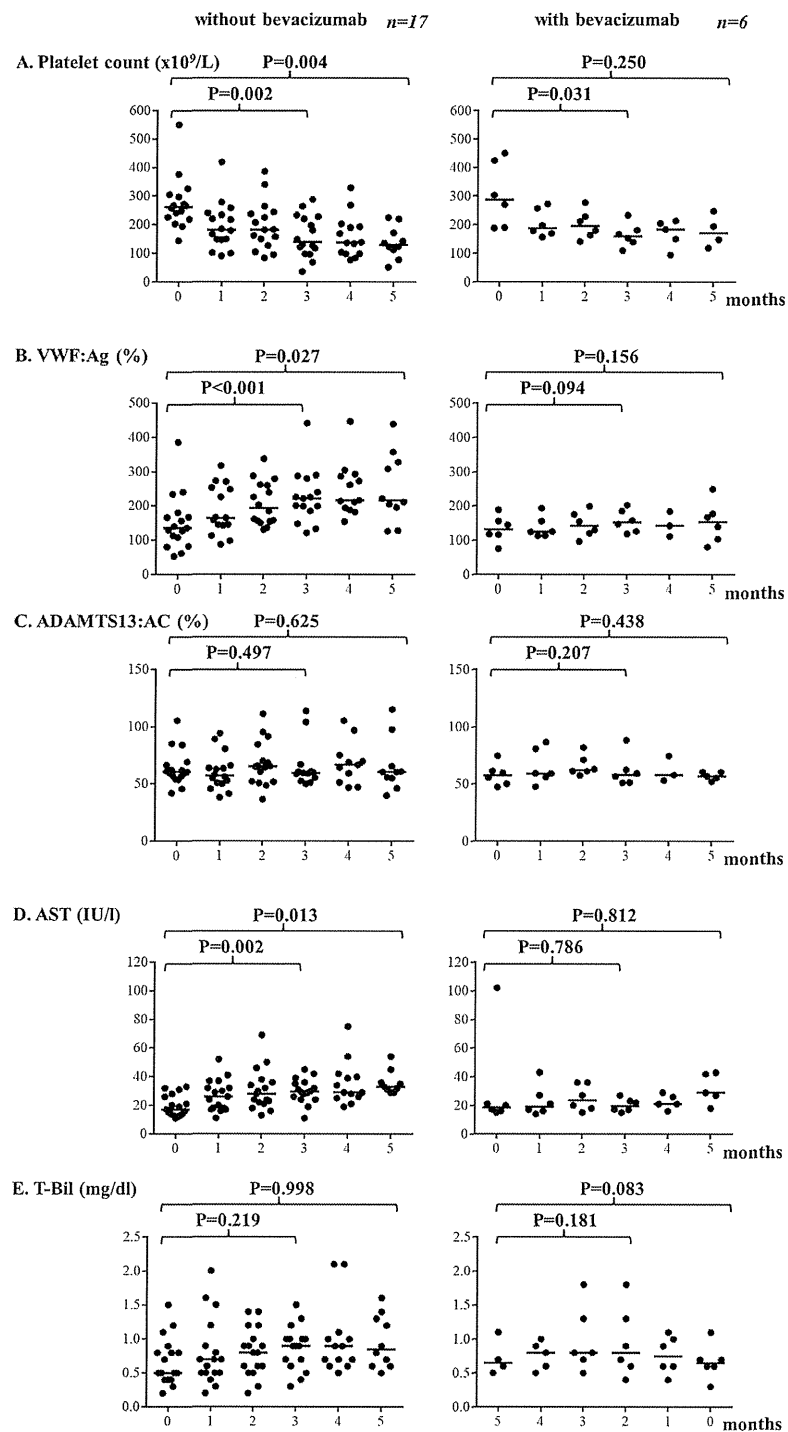
Fig 1. Comparison of platelet count, VWF:Ag, ADAMTS13:AC, AST, and T-Bil between patients treated with and not treated with bevacizumab. (A) Platelet counts decreased until months 3 as the number of chemotherapy cycles increased in both patients who received and did not receive bevacizumab. However, platelet counts in patients not treated with bevacizumab decreased much less in patients who received bevacizumab in months 5. (B) Plasma levels of VWF:Ag increased as the number of chemotherapy cycles

increased in patients not treated with bevacizumab, but did not change in patients treated with bevacizumab. (C) Plasma levels of ADAMTS13:AC were unchanged in both groups. (D) Serum AST levels increased as the number of chemotherapy cycles in patients who did not receive bevacizumab, but they were unchanged in patients with bevacizumab. (E) Plasma levels of T-Bil did not change significantly in either group. VWF:Ag von Willebrand factor antigen, ADAMTS13:AC ADAMTS13 activity, AST Aspartate transaminase, T-Bil total bilirubin.

(P = 0.002 at 3 months, P = 0.013 at 5 months), but were unchanged in patients treated with bevacizumab (Fig 1D). Plasma levels of T-Bil did not change significantly in both groups, but T-Bil levels increased with the number of chemotherapy cycles in patients not treated with bevacizumab, although this difference was not statistically significantly (P = 0.083 at 3 months, P = 0.181 at 5 months) (Fig 1E). A statistical analysis between patients who received or did not receive bevacizumab on each month was performed as shown in S1 Table. Plasma VWF:Ag levels at 2 to 5 months of patients without bevacizumab were significantly lower than those with bevacizumab. In addition, serum AST levels at 3 months of patients who received becacizumab were significantly lower than those who did not received bevacizumab. Consequently, 6 of 17 patients not treated with bevacizumab developed CALI, and there were no cases of CALI among patients treated with bevacizumab.

Comparisons between patients with and without splenomegaly

We analyzed 23 patients with CRC, as shown in Table 1. The spleen size ratio ranged from 0.83 to 2.93 (median, 1.19). Splenomegaly was found in 9 patients (39.1%), who did not have any findings of liver cirrhosis by blood test and CT image. All cases of splenomegaly occurred in patients treated with oxaliplatin-based chemotherapy without bevacizumab (Patients 9–17 in Table 1). On the other hand, no patients who received an oxaliplatin-based regimen with bevacizumab (Patients 18–23) had splenomegaly; the spleen size ratio ranged from 1.00 to 1.37 (median 1.09).

We classified the 23 patients into 2 categories: those with splenomegaly (n = 9) and without splenomegaly (n = 14). As shown in Fig 2A, platelet count decreased as the number of chemotherapy increased in patients with splenomegaly (P = 0.004 at 3 months, P = 0.031 at 5 months) and without splenomegaly (P = 0.002 at 3 months, P = 0.039 at 5 months). These findings are related to bone marrow suppression as a result of chemotherapy. However, thrombocytopenia at 5 months was more pronounced in patients with splenomegaly than in those without splenomegaly (P = 0.005, S2 Table). Plasma levels of VWF:Ag increased with the number of chemotherapy among patients with splenomegaly (P = 0.016 at 3 months, P = 0.006 at 5 months), but not among patients without splenomegaly (Fig 2B, S2 Table). As shown in Fig 2C, plasma levels of ADAMTS13:AC did not change significantly in both groups. Plasma levels of AST increased as the number of chemotherapy cycles increased in patients with splenomegaly (P = 0.009 at 3 months, P = 0.031 at 5 months), but not in patients without splenomegaly (Fig 2D). A statistical analysis between patients with and without splenomegaly on each month was performed as shown in S2 Table. Platelet counts at 2 and 5 months of patients with splenomegaly were significantly lower than those of patients without splenomegaly. Plasma VWF:Ag levels at 2, 4 and 5 months of patients with splenomegaly were significantly higher than those of patients without splenomegaly. Serum AST level at 3 months of patients with splenomegaly were significantly higher than those of patients without splenomegaly.

VWF multimer analysis in patients who did not receive bevacizumab

i) Patients who developed CALI. We performed VWF multimer analysis in 4 representative patients out of 6 patients with CALI who were not treated with bevacizumab (Fig 3). Patient
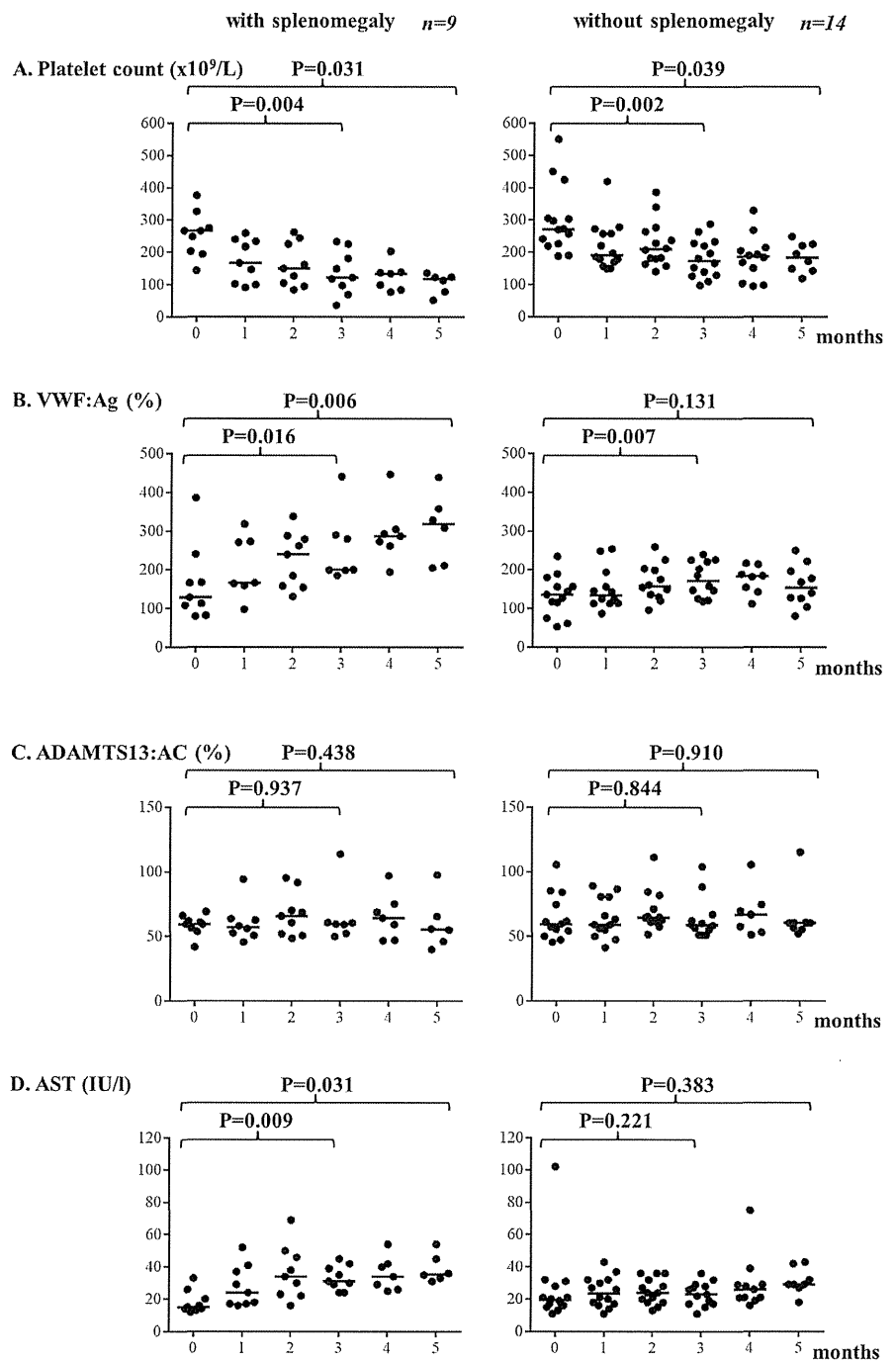
Fig 2. Comparison of platelet count, VWF:Ag, ADAMTS13:AC, and AST between patients with splenomegaly and those without splenomegaly. (A) Platelet counts decreased as the number of chemotherapy cycles increased in patients with and without splenomegaly. (B) Plasma levels of VWF antigen (:Ag) increased as the number of chemotherapy cycles increased in patients with splenomegaly. (C) Plasma levels of ADAMTS13:AC were unchanged in both groups. (D) Plasma levels of aspartate transaminase (AST) increased as the number of chemotherapy cycles increased in patients with splenomegaly, but not in patients without splenomegaly. VWF:Ag von Willebrand factor antigen, ADAMTS13:AC ADAMTS13 activity, AST Aspartate transaminase.

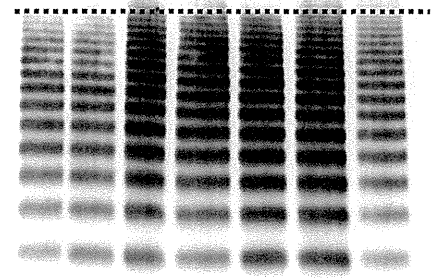doi:10.1371/journal.pone.0143136.g002

Patient.4

UL-VWFM -   -   +   +   +
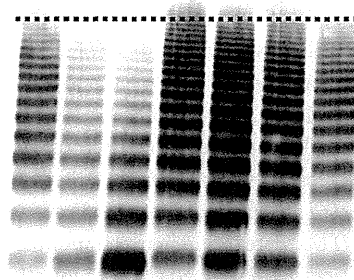
months	NP	0	1	2	3	4	NP
Platelet (10⁹/L)		240	180	180	70	140	
ALT (IU/L)		30	23	28	90	38	
T-Bil (mg/dl)		0.4	0.5	0.8	0.6	0.7	
VWF:Ag (%)		62	87	161	299	214	
VWF:CB(%)		56	43	347	294	261	
ADAMTS13:AC(%)		58	50	64	66	67	
VWF:CB/ADAMTS13:AC		1.0	0.9	5.4	4.5	3.9	

Patient.11

-   +   -   -   +

NP	0	1	2	3	4	NP
	270	260	230	230	230	
	28	31	65	88	39	
	0.8	1.1	1.2	1.2	1	
	80	137	159	184	201	
	169	247	250	253	278	
	61	59	63	51	61	
	2.8	4.2	1.2	1.2	4.6	

Patient.16

UL-VWFM -   +   +   +   +   -
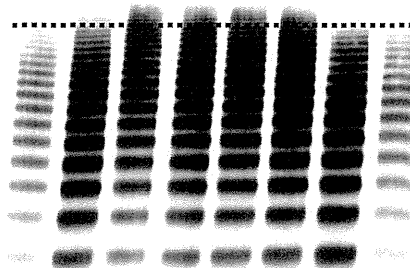
months	NP	0	1	2	3	4	5	NP
Platelet (10⁹/L)		190	190	140	140	140	110	
ALT (IU/L)		7	8	21	18	20	19	
T-Bil (mg/dl)		1.2	1.2	0.9	1.1	2.2	1.3	
VWF:Ag (%)		386	226	338	262	358	265	
VWF:CB (%)		111	488	434	438	443	235	
ADAMTS13:AC(%)		69	60	70	75	66	73	
VWF:CB/ADAMTS13:AC		1.6	8.1	6.2	5.8	6.7	3.2	

Patient.17

+   -   -   -   +   +

NP	0	1	2	3	4	5	NP
	330	100	80	70	80	80	
	19	27	29	23	35	32	
	0.5	0.7	0.8	1.5	2.1	2.3	
	166	271	131	280	305	328	
	289	155	27	195	234	498	
	60	94	92	114	97	98	
	4.8	1.6	0.3	1.7	2.4	5.1	

Fig 3. VWF multimer analysis in patients with CALI who were not treated with bevacizumab. VWF multimer analysis was performed in 4 representative patients out of 6 patients with CALI not treated with bevacizumab. These patients developed CALI during month 3 or 4. UL-VWFMs were found before and during CALI in all patients. Decreased levels of H-VWFMs were found in Patient 4 at months 0 and 1, and in Patient 17 at month 2. VWF von Willebrand factor, CALI chemotherapy-associated liver injury, UL-VWFMs unusually-large VWF multimers, H-VWFM high molecular weight VWF multimers, AST aspartate transaminase, T-Bil total bilirubin, VWF:Ag VWF antigen, VWF:CB VWF collagen binding activity.

4 developed CALI in 3 months after starting chemotherapy. Platelet count dropped sharply to 70×109/L in month 3, when CALI developed. H-VWFM levels were decreased in months 0 and 1. UL-VWFMs appeared in months 2, 3, and 4. Plasma VWF:Ag levels were relatively low in months 0 and 1, and increased up to 299% in month 3. Plasma levels of ADAMTS13:AC were

within the normal range (>50% of normal). VWF:CB levels and the ratio of VWF:CB to ADAMTS13:AC was sharply increased after month 2 at the same time as UL-VWFM appeared. There was no instances of splenomegaly after the start of chemotherapy. Patient 11 was diagnosed with CALI in month 3. Platelet counts remained in the normal range. Plasma levels of VWF:Ag gradually increased along with the number of chemotherapy cycles. UL-VWFMs were found in months 1 and 5, when the ratios of VWF:CB to ADAMTS13:AC were clearly high values. He developed splenomegaly during chemotherapy. Patient 16 was diagnosed with CALI in month 4. Platelet count gradually decreased along with the number of chemotherapy cycles. He had extremely high VWF:Ag levels even in month 0, but VWF:CB level was normal and UL-VWFMs were not detected at this point. Both VWF:Ag and VWF:CB levels remained continuously high and UL-VWFMs were found between months 1 and 4. Splenomegaly was observed in this patient. Patient 17 developed CALI in month 4. His platelet count decreased during chemotherapy. Both VWF:Ag and VWF:CB levels were relatively high, with UL-VWFMs detected in month 0. His VWF:Ag level increased but VWF:CB decreased during month 1 without UL-VWFMs. However, in month 2 both VWF levels suddenly dropped and H-VWFMs disappeared. Subsequently, H-VWFM levels increased and UL-VWFMs were found in months 4 and 5. This patient developed splenomegaly during chemotherapy. All 4 patients who developed CALI had UL-VWFMs before and at the time of CALI diagnosis.

    ii) Patients who did not develop CALI. Results from VWF multimer analysis in 4 representative patients out of 11 patients without CALI who were not treated with bevacizumab are shown in Fig 4. In Patient 7, his platelet count dropped slightly in months 2 and 4. Plasma levels of VWF:Ag increased after the start of chemotherapy. However, UL-VWFMs were not found during chemotherapy. He did not develop splenomegaly after chemotherapy. In Patient 10, his platelet count was low during the start of chemotherapy. He developed splenomegaly after chemotherapy. VWF:Ag levels increased 2 months after starting chemotherapy. VWF multimer analysis showed lower levels of H-VWFs in month 1, which subsequently increased. UL-VWFMs were found in month 3, but they decreased again in months 4 and 5. The change of VWF:CB levels paralleled with the levels of H-VWF and UL-VWFM. He developed splenomegaly after chemotherapy. In Patient 14, platelet counts decreased gradually with the number of chemotherapy cycles. Both levels of VWF:Ag and VWF:CB were elevated after the start of chemotherapy. UL-VWFMs were found in months 1, 2, 4 and 5. In Patient 15, platelet count decreased in months 1, 2, and 5. Plasma VWF:Ag levels increased after month 2. UL-VWFMs were found in months 2 and 4 with the elevation of VWF:CB. Splenomegaly was observed in this patient. In this group, UL-VWFMs were found during chemotherapy in patients with splenomegaly, except for Patient 7.

## VWF multimer analysis in patients treated with bevacizumab

S1 Fig shows the VWF multimer analysis results in 4 representative patients out of 6 patients treated with bevacizumab. No patient who received bevacizumab developed both CALI and splenomegaly during chemotherapy. The platelet count in these patients was maintained at almost normal levels. All 4 patients had nearly normal levels of VWF:Ag. Moreover, no patients had any apparent abnormalities in VWF multimer distribution, including the lack of H-VWFMs and the appearance of UL-VWFMs. However, VWF:CB levels were relatively high compared with VWF:Ag levels.

Histopathological evidence of sinusoidal obstruction in the liver

We performed liver sections to evaluate for metastatic liver injury during chemotherapy in 4 patients. One patient had Grade 0 sinusoidal congestion, one patient had Grade 1, and 2
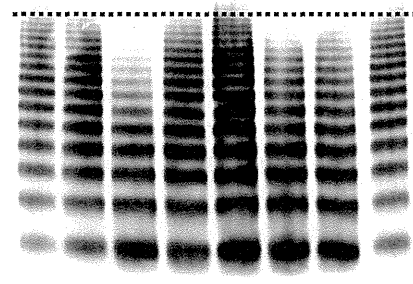
Patient.7

UL-VWFM − − − − −
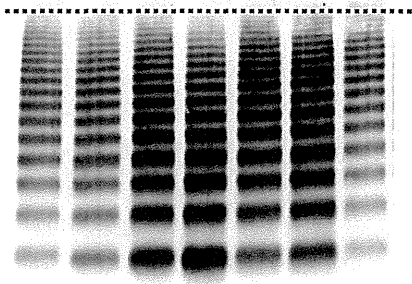
months	NP	0	1	2	3	4	NP
Platelet (10⁹/L)		270	220	120	230	140	
ALT (IU/L)		39	19	16	14	16	
T-Bil (mg/dl)		1.5	0.9	0.8	0.9	1	
VWF:Ag (%)		127	254	212	226	224	
VWF:CB(%)		132	195	122	194	228	
ADAMTS13:AC(%)		62	63	64	51	43	
VWF:CB/ADAMTS13:AC		2.1	3.1	1.9	3.8	5.3	

Patient.10

− − − + − −

NP	0	1	2	3	4	5	NP
	200	90	100	40	80	50	
	6	10	13	12	11	20	
	0.9	0.7	0.8	1.2	0.6	0.7	
	129	111	280	441	220	212	
	115	80	137	450	83	150	
	59	56	52	59	64	55	
	1.9	1.4	2.6	7.6	1.3	2.7	

Patient.14

UL-VWFM − + + − + −
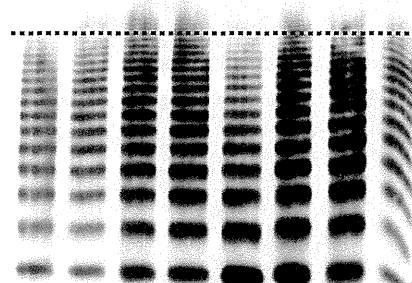
months	NP	0	1	2	3	4	5	NP
Platelet (10⁹/L)		380	220	160	120	130	120	
ALT (IU/L)		15	34	33	23	29	27	
T-Bil (mg/dl)		0.3	0.4	0.6	0.7	1	1.1	
VWF:Ag (%)		108	273	288	185	447	313	
VWF:CB(%)		139	395	427	124	343	152	
ADAMTS13:AC(%)		54	51	61	52	69	46	
VWF:CB/ADAMTS13:AC		2.6	7.7	7.0	2.4	5.0	3.3	

Patient.15

− − + − + −
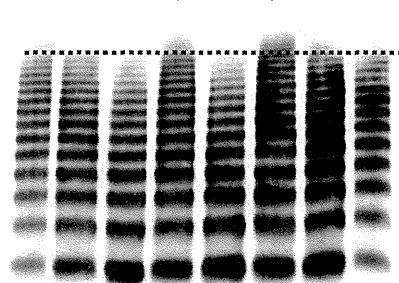
NP	0	1	2	3	4	5	NP
	270	100	100	200	200	70	
	9	8	10	10	14	13	
	0.5	0.5	0.5	0.6	0.5	0.6	
	167	164	239	290	293	309	
	272	71	233	85	291	235	
	62	58	69	60	59	56	
	4.4	1.2	3.4	1.4	4.9	4.2	

Fig 4. VWF multimer analysis in patients without CALI who were not treated with bevacizumab. Results of VWF multimer analysis in 4 representative patients out of 11 patients without CALI not treated with bevacizumab are shown. UL-VWFMs were found in Patients 10, 14, and 15, who did not develop CALI. In Patient 10, decreased levels of H-VWFMs were observed during months 1 and 4. VWF von Willebrand factor, CALI chemotherapy-associated liver injury, UL-VWFMs unusually-large VWF multimers, H-VWFM high molecular weight VWF multimers, AST aspartate transaminase, T-Bil total bilirubin, VWF: Ag VWF antigen, VWF:CB VWF collagen binding activity.

patients had Grade 2. Consecutive slices in the same patient immunohistochemically stained with platelet-specific anti-IIb/IIIa, anti-VWF, anti-fibrin antibodies demonstrated Grade 1 and 2 SOS. As shown in Fig 5a and 5b, Patient 4's liver tissue demonstrated extensive Grade 2 sinusoidal dilatation and platelet thrombi in the liver sinusoids. Many of these thrombi were positive for both IIb/IIIa and VWF (Fig 5d and 5e), indicating that they were platelet thrombi. Some thrombi were positive for fibrin (Fig 5f). These results indicated that sinusoidal congestion mainly resulted from platelet thrombi in the liver sinusoids.
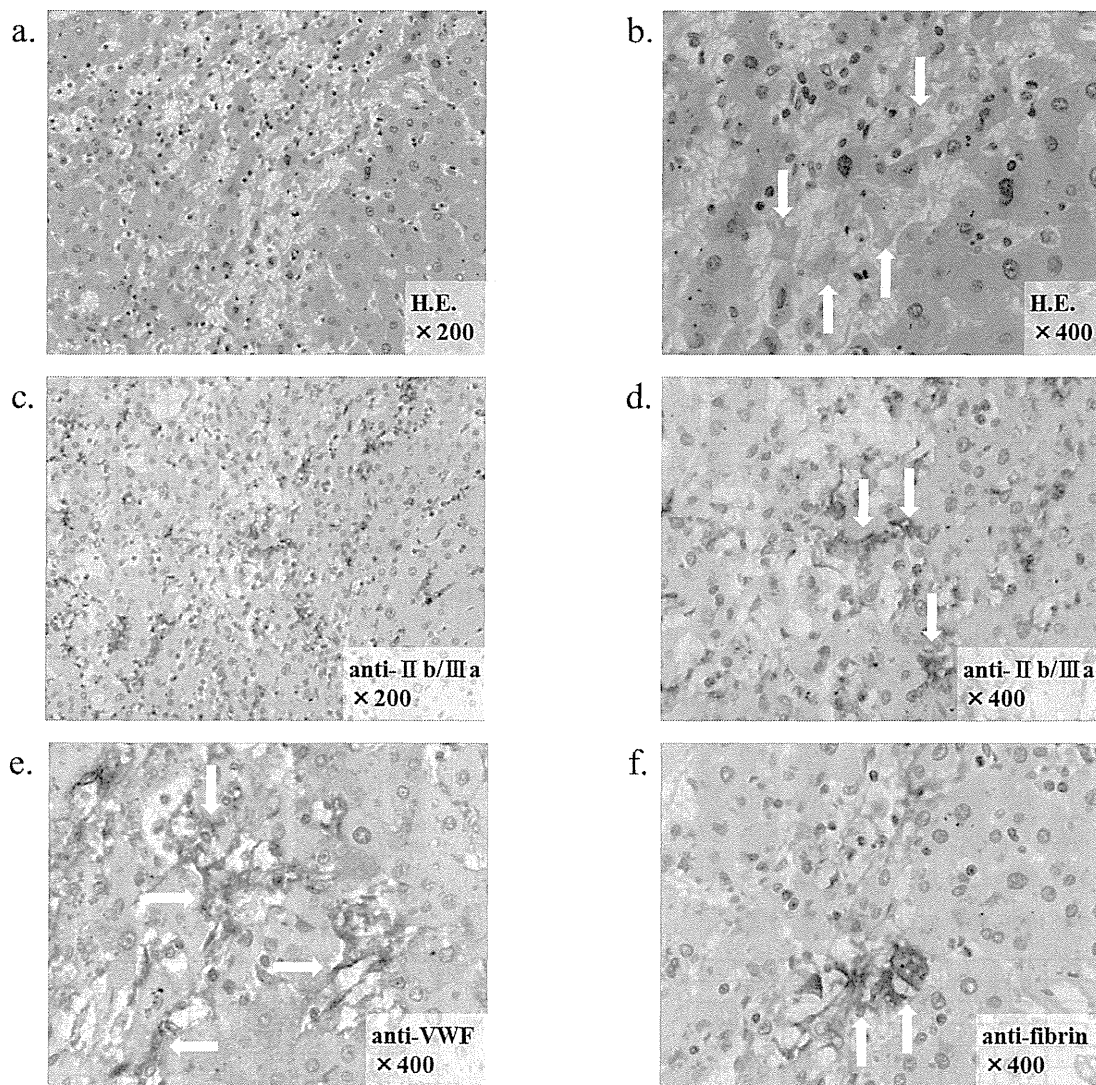
Fig 5. Immunohistochemical analysis of liver specimens in patients with SOS. Histological findings in the liver with hematoxylin and eosin (H. E.) staining in Patient 4 included extensive Grade 2 sinusoidal dilatation (A) and platelet thrombi in the liver sinusoids, as indicated with white arrows (B). Many of these thrombi were positive for both platelet-specific anti-IIb/IIIa (C, D) and anti-VWF (E), which showed that they are platelet thrombi, as indicated with arrows. Some thrombi were positive for fibrinogen (F), which indicated that they are fibrinogen thrombi, but there were much less frequently observed than platelet thrombi.

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Discussion

The introduction of oxaliplatin-based chemotherapy contributed to a significant improvement in prognosis among patients with CRC. However, liver injury, including SOS, has been observed after chemotherapy containing oxaliplatin, which is called "blue liver" by hepatic surgeons [22]. The precise mechanism of liver injury due to oxaliplatin-based chemotherapy remains unclear. However, oxaliplatin is more toxic to sinusoidal endothelial cells than hepatocytes [23]. In addition, increases in spleen size and decreases in platelet count were commonly observed in patients with CRC on oxaliplatin-based chemotherapy [8].

Two hypotheses have been proposed to explain the mechanism underlying oxaliplatin-induced thrombocytopenia [7, 8]. One is bone marrow suppression by chemotherapy and the other is splenic sequestration of platelets secondary to portal hypertension. The first hypothesis is not specific for oxaliplatin and cannot explain the association among thrombocytopenia, SOS and splenomegaly. We found differences in the extent of platelet count decreases after chemotherapy between patients who received and did not receive bevacizumab (Fig 1A). Bevacizumab did not block myelosuppression associated with chemotherapy. The second mechanism could explain the relationship between splenomegaly and thrombocytopenia in addition to SOS. Based on our previous study of patients with SOS after SCT, we hypothesize that the second mechanism is related to VWF-rich platelet thrombosis in sinusoids, which induces portal hypertension and splenomegaly.

To the best of our knowledge, this is the first study analyzing the levels of VWF:Ag, activity and the appearance of UL-VWFMs in CRC patients with CALI from oxaliplatin-based chemotherapy. In this study, we chose VWF:CB as VWF activity, since this method appear to be reproducible and sensitive. Collagen binding assay is based on the physiological principle of the interaction between VWF and collagen. The adhesive activity of VWF depends the molecular size of VWF [24]. Therefore, VWF:CB usually increase with the molecular size of VWF. As shown in Figs 3 and 4, VWF:CB levels showed good correlation with the appearance of UL-VWFM. However, as shown in S1 Fig, VWF:CB levels were high even in the plasmas with the lack of UL-VWFM. These observations that high level of VWF:CB was found even in the sample with normal VWF multimers were reported in previous study [25]. This might be because the evaluation of VWF activity was difficult in one method.

Based on the results of this study, we speculate that thrombocytopenia is mainly caused by platelet consumption in platelet thrombi, and splenomegaly is caused by the occlusion of liver sinusoids.

SOS is a well-known life-threatening complication of SCT, which is clinically diagnosed by the triad of hepatomegaly, ascites, and hyperbilirubinemia [26]. It is histologically characterized by sinusoidal dilatation, congestion, and nodular regenerative hyperplasia [27]. The sinusoidal endothelial cell is also suspected to be the primary site of toxic injury from chemotherapy and/or radiation before SCT. Severe endothelial cell damage results in the release of UL-VWFMs from endothelial cells [14]. We have reported that levels of high to intermediate VWFMs were decreased during the early post-SCT phase, but UL-VWFMs appeared just before VOD onset [13]. The most important function of VWF is to act as the molecular glue for platelet adhesion and aggregation at sites of vascular injury. VWF binding to platelets through glycoprotein 1b depends on its molecular weight. Therefore, UL-VWFMs are the most active form for platelet thrombus formation. VWF is exclusively produced in endothelial cells and stored in Weibel-Palade bodies (WPBs) in endothelial cells [28]. In response to a variety of agonists such as thrombin, histamine, VEGF, serotonin, epinephrine, and vasopressin, VWF is secreted into the circulation [29]. The vasopressin analogue desmopressin is used to increase plasma VWF levels in the treatment of von Willebrand disease [30]. There are 2 mechanisms of VWF secretion from endothelial cells. One is release from severely injured endothelial cells, and the other is exocytosis of WPBs by endothelial cells stimulated by various cytokines.

In this study, plasma levels of ADAMTS13:AC were not decreased (>50% of normal) during oxaliplatin-based chemotherapy. We have reported that plasma levels of ADAMTS13:AC were decreased in patients with SCT-associated SOS [12]. The lowest values of ADAMTS13:AC were found 14 days after SCT [12, 13]. ADAMTS13 is exclusively synthesized by stellate cells in the liver [31]. Chemotherapy and/or radiation associated with SCT damages stellate cells, resulting decreased ADAMTS13:AC. However, oxaliplatin-based chemotherapy might

cause much less damage to stellate cells in the liver. To study short-term changes in factors, monthly examinations might be insufficient. Therefore, we could not precisely characterize changes in plasma ADAMTS13 activity levels.

Ribero et al [11] reported that oxaliplatin plus bevacizumab was oncologically more effective than oxaliplatin alone and the incidence of hepatic injury was lower when bevacizumab was used with oxaliplatin. The effects of bevacizumab against tumors include the regulation of angiogenesis and improved delivery of chemotherapy [9]. However, it is unclear how bevacizumab protects from hepatic injury in patients treated with oxaliplatin-based chemotherapy. VEGF is reported to activate endothelial exocytosis of WPBs, which leads to the release of VWF from endothelial cell [32]. Recently, VEGF was identified as a strong promoter of endothelial cell activation accompanied by UL-VWFM release in tumor microvessels [33]. We speculated that VEGF might be one of the causative factors for CALI and SOS after oxaliplatin-based chemotherapy. In our patients, VWF levels might increase due to both endothelial cell activation by VEGF and the sinusoidal endothelial cell damage by oxaliplatin-based chemotherapy. Therefore, VWF levels together with UL-VWFM increase in blood circulation especially in sinusoids. As consequence, VWF-rich platelet thrombi was made in sinusoids and resulted in the development of CALI and SOS. Bevacizumab, an anti-VEGF monoclonal antibody, reduces plasma levels of VEGF, and thereby lowers plasma levels of VWF to some extent as shown in Fig 1 and S1 Table. In fact, one study reported that plasma VEGF levels before a conditioning regimen were significantly higher in patients with SCT-associated SOS than in patients without SOS [34].

Although this study used a novel approach to study liver injury due to oxaliplatin-based chemotherapy in patients with CRC, there are some limitations. First, the number of patients analyzed in this study was relatively small. Therefore, sufficient statistical analysis was not possible. Second, the blood samples were collected once a month and prior to the administration of chemotherapy. Since the factors analyzed in this study seemed to change over a short period of time, we might have missed the correct sequence of change in these factors. Finally, pathological examination of liver tissue to diagnose SOS was performed only in 4 patients. Of these, 2 patients had evidence of Grade 2 SOS (Patients 4 and 7). Patient 4 developed CALI and SOS confirmed by the presence of VWF-rich platelet thrombi in the sinusoids (Fig 5). This is regarded as a convincing cause of CALI. However, Patient 7 had Grade 2 SOS, but not CALI. This might have been due to the timing of blood samples or different pathophysiological mechanisms for CALI and SOS. Conversely, one patient (Patient 11) had CALI, but not SOS. This might be due to the timing of liver resection or patchy SOS findings in the part of the liver examined. It would be necessary to perform pathological examinations in all patients treated with oxaliplatin-based chemotherapy to confirm the existence of SOS.

In conclusion, we found an association between VWF and liver injury, including SOS, in CRC patients treated with oxaliplatin-based chemotherapy. VWF-rich platelet thrombi in liver sinusoids due to UL-VWFMs secreted as a result of endothelial injury might be one cause of CALI associated with oxaliplatin-based chemotherapy. Bevacizumab could protect against CALI associated with oxaliplatin-based chemotherapy through lowering plasma levels of VWF.

Supporting Information

S1 Fig. VWF multimer analysis in patients treated with bevacizumab. VWF multimer analysis was performed in 4 representative patients out of 6 patients treated with bevacizumab. None of the patients developed both CALI and splenomegaly during chemotherapy. All 4 patients had nearly normal levels of VWF:Ag. UL-VWFMs were not observed except at month

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0 in Patient 22. VWF von Willebrand factor, CALI chemotherapy-associated liver injury, UL-VWFMs unusually-large VWF multimers, H-VWFM high molecular weight VWF multimers, AST aspartate transaminase, T-Bil total bilirubin, VWF:Ag VWF antigen, VWF:CB VWF collagen binding activity.
(TIF)

S1 Table. Comparison between patients who received or did not receive bevacizumab.
(DOCX)

S2 Table. Comparisons between patients with and without splenomegaly.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: NN MM YF YN. Performed the experiments: NN MH KH. Analyzed the data: NN SK. Contributed reagents/materials/analysis tools: FK SK. Wrote the paper: NN MM YF YN.

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ORIGINAL ARTICLE

Efficacy and safety of rituximab in Japanese patients with relapsed chronic immune thrombocytopenia refractory to conventional therapy

Yoshitaka Miyakawa[1] · Shinya Katsutani[2] · Takahiro Yano[3] · Shosaku Nomura[4] ·
Kaichi Nishiwaki[5] · Yoshiaki Tomiyama[6] · Masaaki Higashihara[7] · Yukari Shirasugi[8] ·
Masakatsu Nishikawa[9] · Katsutoshi Ozaki[10] · Takayuki Abe[11] · Kayoko Kikuchi[11] ·
Yuzuru Kanakura[12] · Kingo Fujimura[13] · Yasuo Ikeda[14] · Shinichiro Okamoto[15]

Abstract Primary immune thrombocytopenia (ITP) is an autoimmune disease mediated by the production of auto-antibody against platelets. Rituximab, an anti-CD20 antibody, is reported to be useful for treatment of ITP. In Japan, however, robust evidence on this treatment has not been accumulated. Hence, we conducted this open-label phase III clinical trial to confirm the efficacy and safety of rituximab, administered at 375 mg/m^2 once per week at weekly intervals for 4 consecutive weeks in Japanese patients with chronic ITP, who had relapsed and were refractory to conventional therapy. The primary endpoint was defined as the percentage of patients with a platelet count above 50 × 10^9/L at week 24 after the first dose of rituximab, which was 30.8 % of 26 patients (95 % confidence interval 14.3–51.8 %). Although the lower confidence limit of primary endpoint failed to meet the pre-specified threshold of 20 %, the clinical efficacy of rituximab is substantial in consideration of the 2 % response rate in the placebo arm in other clinical studies in patients with chronic ITP. We conclude that rituximab is clinically useful and safe in the treatment of Japanese patients with chronic ITP, achieving the goal of maintaining platelet count and reducing risk of bleeding while minimizing treatment-related toxicity.

Keywords Immune thrombocytopenia · Platelets · Rituximab

✉ Yoshitaka Miyakawa
miyakawa@saitama-med.ac.jp

[1] Department of General Internal Medicine, Saitama Medical University, 38 Morohongo, Moroyamamachi, Iruma-gun, Saitama 350-0495, Japan

[2] Department of Hematology and Oncology, Hiroshima University, Hiroshima, Japan

[3] Hematology, Internal Medicine, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

[4] First Department of Internal Medicine, Kansai Medical University Hirakata Hospital, Osaka, Japan

[5] Department of Oncology and Hematology, The Jikei University School of Medicine, Kashiwa Hospital, Chiba, Japan

[6] Department of Blood Transfusion, Osaka University Hospital, Osaka, Japan

[7] Department of Hematology, Kitasato University Hospital, Kanagawa, Japan

[8] Department of Hematology and Oncology, Tokai University Hospital, Kanagawa, Japan

[9] Clinical Research Support Center, Mie University Hospital, Mie, Japan

[10] Division of Hematology, Jichi Medical University, Tochigi, Japan

[11] Center for Clinical Research, Keio University School of Medicine, Tokyo, Japan

[12] Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Osaka, Japan

[13] Yasuda Women's University, Hiroshima, Japan

[14] Faculty of Science and Engineering, Waseda University, Tokyo, Japan

[15] Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

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Introduction

Primary immune thrombocytopenia (ITP) is a thrombocytopenia-causing autoimmune disease. Approximately 20,000 patients are suffered from this disease in Japan, and about 3000 new cases occur per year [1]. The causes of decrease in the platelet count are known to be destruction of platelets, to which auto-antibody is attached, in the spleen, and defects of proliferation and maturation of megakaryocyte due to the relative shortage of thrombopoietin (TPO). The major clinical symptoms and signs of ITP are petechiae and mucosal hemorrhage. ITP is classified by duration into newly diagnosed (within 3 months of onset), persistent (3–12 months' duration) and chronic (12 months or more in duration). Of ITP with onset in adult age, about 90 % of the patients are chronic and its male-to-female ratio is 1:2. Massive hemorrhage is relatively uncommonly seen in chronic ITP, whereas seen in the other types of ITP.

Patients who show no effects on standard therapy and have platelet count $\leq 30 \times 10^9$/L are diagnosed with refractory ITP, and those are about 10 % of total patients with ITP. Such patients have a 4.2-fold higher risk of death than healthy population [2]. In Japan, nearly half of patients with ITP are infected with *Helicobacter pylori*, and *H. pylori* eradication therapy is effective for restoring the platelet count in 60 % of patients [3].

The first-line treatment of standard therapy is corticosteroid administration. Corticosteroid administration can only be discontinued in about 10–20 % of the patients. For most of the patients, the steroid therapy is continued especially in Japan. Splenectomy is chosen as a second-line treatment for patients who fail to respond or have poor tolerability to corticosteroids. A radical cure can be expected from splenectomy in about 70 % of patients, while for the remaining 30 % of patients this surgical intervention makes to be ineffective. Splenectomy has several concerns such as complications in the perioperative phase (mortality rate at 0.1 % and complication rate at 10 %), postoperative depression of immune functions, and relapses in about 20 % of patients [4]. Since it is difficult to predict the efficacy of surgical removal of the spleen in patients with ITP, both patients and physicians tend to avoid splenectomy in Japan, as well as in Europe and the United States. Non-responders to splenectomy die at about 10 % from serious hemorrhage such as cerebral hemorrhage. Therefore, the goal of ITP treatment is to increase the platelet count to $\geq 30 \times 10^9$/L to avoid such fatal bleeding [5, 6].

Immunosuppressants such as azathioprine and cyclosporine, and anticancer agents such as cyclophosphamide and vincristine, in off-label use, are empirically prescribed for patients who are non-responding or ineligible for splenectomy. With these drugs, response rates are as low as about 30 %, and adverse events are rather frequently

observed. Recently, thrombopoietin receptor agonists (romiplostim and eltrombopag) have been approved for refractory ITP and showed efficacy in nearly 60 % of patients. However, some concerns arise from these agonists, including offset of drug effect back to baseline level in about 2 weeks after discontinuation of the medication, a high cost of drug expense as high as 2–3 million yen per year, a high incidence of thrombotic complications in some of patients with a certain background, and disease progression to myelofibrosis or acute leukemia after long-term treatment.

Rituximab is a chimeric monoclonal antibody against the CD20 antigen and prepared by recombinant DNA technology. Rituximab was approved in Japan for the treatment of B cell non-Hodgkin's lymphoma, microscopic polyangiitis (MPA), and granulomatous polyangiitis (GPA; Wegener granulomatosis). In addition to these indications, this drug was approved for the treatment of chronic lymphoid leukemia and rheumatoid arthritis in the United States and Europe. Rituximab specifically eliminates CD20-positive B lymphocytes; therefore, its efficacy for various disorders relevant to B cell abnormalities is anticipated [7, 8]. Recent studies have demonstrated that B cells are involved in the onset and maintenance of autoimmune diseases, and the efficacy of treatment with rituximab has been reported in autoimmune disorders such as systemic lupus erythematosus (SLE), multiple sclerosis, nephrotic syndrome, and thrombotic thrombocytopenic purpura (TTP). Outside of Japan, rituximab is extensively prescribed and accepted as the second-line treatment for refractory ITP [5, 9–11]. A systematic review on the efficacy and safety of rituximab in approximately 300 patients with ITP by Arnold et al. [11] showed that the response rate was 62.5 %, and the time to therapeutic response was 5.5 weeks. A phase II clinical trial of rituximab in 60 patients with refractory ITP in France showed that the response rate was 40 % after 1 year of the treatment [12]. As seen in a number of reports outside Japan, the efficacy and safety of rituximab in patients with refractory ITP have been extensively evaluated and the effectiveness of the drug has been demonstrated.

In Japan, there are several case reports to indicate the efficacy of rituximab in the treatment of refractory ITP, but robust evidence on this treatment has not yet been accumulated such as from clinical studies. Rituximab is then prescribed off-label for rescuing patients with refractory ITP, but the medication cost of rituximab is not reimbursed under the Japanese National Health Insurance program.

Hence, we conducted this open-label phase III clinical trial to confirm the efficacy and safety of rituximab, administered at 375 mg/m^2 (body surface area) once a week, at weekly intervals for 4 consecutive weeks in Japanese patients with chronic refractory ITP. This study was implemented in accordance with the International

Consensus Guidelines for Diagnosis and Treatment of ITP [10], and the Japanese Guidelines for Treatment of ITP. This study was registered with the Japan Medical Association Center for Clinical Trials (JMACTR; CTR Number: JMA-IIA00070, https://dbcentre3.jmacct.med.or.jp/jmactr/default.aspx?JMACCTID=JMA-IIA00070).

Materials and methods

Patient population

Patients included in this study were: Japanese, aged ≥20 years, and diagnosed with chronic refractory ITP at least 12 months before the enrollment of this study. The definition of the term *refractory* in this study was as follows: platelet counts ≤30 × 10^9/L (measured at weeks 4 and 2 before enrollment), ineffective or intolerable for steroids, ineffective or judged as inappropriate by investigators for splenectomy, and ineffective, intolerable or judged as inappropriate by investigators for thrombopoietin receptor agonists.

Study design

This study was an open-label multicenter phase III clinical trial conducted between October 2011 and July 2013 in ten clinical institutions in Japan. The study consisted of screening (4 weeks), treatment (4 weeks), and follow-up periods (20 weeks).

Rituximab was administered at 375 mg/m^2 in once a week consecutively for 4 weeks (weeks 0, 1, 2, and 3). To prevent infusion reactions associated with rituximab infusion, patients received pre-medications of oral antipyretic–analgesics, oral antihistamines, and intravenous hydrocortisone at 30 min before each administration of rituximab.

During the study period, the following concomitants or therapies were prohibited: immunoglobulin preparations, drugs which stimulate platelet production, splenectomy, *H. pylori* eradication therapy, hematopoietic factors, antineoplastics and platelet transfusion.

Efficacy and safety analysis

The primary efficacy endpoint was a response rate: the percentage of patient with the platelet count ≥50 × 10^9/L at 24 weeks after the first administration of the study drug.

The major secondary efficacy endpoints included the percentage of patients with the platelet count ≥100 × 10^9/L and who did not have bleeding at week 24, the percentage

of patients with the platelet count ≥30 × 10^9/L and ≥twofold higher than the baseline value and who did not have bleeding at week 24, and the improvement rate of bleeding symptoms [World Health Organization (WHO) bleeding scale]. In addition, the changes of peripheral blood B cells (CD19 and CD20) and T cells (CD3), and changes of serum IgG, IgM, and IgA levels were evaluated as exploratory endpoints. Safety parameters (adverse events and clinical laboratory data) were also assessed.

Statistical considerations

Sample size and its rationale were pre-specified in the study protocol. Response rate in the primary endpoint was assumed to be 50 % based on results of clinical studies [11, 12]. Twenty-four patients were needed to have ≥80 % power to show that the lower limit of 95 % confidence interval (CI) for the response rate is greater than a threshold (20 %). The threshold was determined conservatively taking account of a response rate, 2 % (1/42 patients, 95 % CI 0–12.6 %) in placebo group in the phase III clinical trial of romiplostim in patients with refractory ITP [13]. All enrolled patients were included in the primary efficacy analysis population (full analysis set, FAS). Demographic factors and baseline characteristics were summarized with mean ± standard deviation (SD) or median (interquartile range, IQR) depending on distributions. Exact 95 % CIs for proportions were calculated with the Clopper–Pearson method. Paired proportions were compared with the exact McNemar's test. For continuous valuables, values at each time point were compared with baseline values by signed rank sum test. Significance level was a two-sided 5 % for all tests. All data were analyzed with SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

Ethical considerations

This clinical trial was conducted in compliance with the ethical principles of the Declaration of Helsinki, the Japanese Guidelines for Good Clinical Practice, and other relevant regulatory requirements. The investigator or co-investigator gave a full explanation of the clinical trial to patients prior to participation in the study and, upon confirming that the patients gained a good understanding of the nature of the study, obtained written informed consent for voluntary participation in the study. Prior to conduct of this clinical trial, the institutional review board (IRB) of each participating medical facility reviewed the ethical, scientific, and medical propriety of this clinical trial and approved this study.

Table 1 Patient demography and disease characteristics

	Analysis set: $n = 26$
Sex (females)[a]	23 (88.5 %)
Age (years)[b]	39.7 ± 13.0 (23, 69)
Body weight (kg)[b]	56.5 ± 9.7 (37.9, 73.1)
Duration of ITP (years)[c]	5.9 (1.9, 11.2)
Hemorrhagic symptoms (WHO bleeding scale)	
Grade 0[a]	11 (42.3 %)
Grade 1[a]	14 (53.8 %)
Grade 2[a]	1 (3.8 %)
Baseline platelet count (10^9/L)[c]	22 (17, 24)
Baseline CD3 cells (/μL)[c]	1035.5 (798, 1588)
Baseline CD19 cells (/μL)[c]	97 (63, 147)
Baseline CD20 cells (/μL)[c]	91.5 (59, 145)
Had splenectomy (yes)[a]	4 (15.4 %)
Had H. pylori eradication (yes)[a]	9 (34.6 %)
Had complications (yes)[a]	21 (80.8 %)
Previous therapy for ITP	
Had corticosteroids (yes)[a]	18 (69.2 %)
Had high-dose immunoglobulin therapy (yes)[a]	10 (38.5 %)
Had thrombopoietin receptor agonists (yes)[a]	7 (26.9 %)
Number of previous therapies for ITP[c]	2 (1, 3)

[a] Number of patients (%)

[b] Mean ± standard deviation (range)

[c] Median (25 % point, 75 % point)

Results

Patient characteristics

Written informed consent was obtained from 49 patients in this clinical trial. Of them, 26 patients who met the inclusion criteria were enrolled. All the 26 patients completed a total of four doses of rituximab infusion and were included in the FAS. None of the patients discontinued the study treatment. The following measured values of the platelet count were partially excluded from the FAS: the platelet counts of one patient at week 4 and week 0 when platelet aggregation was seen in the sample of the patient, and the platelet count of other patient at the time when the platelet count was increased due to emergency treatment (high-dose immunoglobulin therapy plus platelet transfusion) at week 2.

Although protocol deviations were seen in 12 patients, none were relevant to patient eligibility or discontinuation criteria. None of these patients were excluded from the efficacy and/or safety analysis, as the deviations in this study were examined at the case-conference meeting and judged not to significantly influence on the evaluation of the study.

Most of the patients with refractory ITP enrolled in this study were female (88.5 %). The mean age was 39.7 ± 13.0 years (Table 1). The median duration of ITP was 5.9 years (IQR 1.9–11.2), and Grade 0, 1, and 2 hemorrhagic symptoms in severity at baseline were 42.3, 53.8, and 3.8 % of patients, respectively. The median platelet count at baseline was 22 × 10^9/L (IQR 17–24). Of the enrolled patients, 15.4 % had previously received splenectomy and 34.6 % underwent H. pylori eradication. The percentages of patients who had previously received corticosteroids, high-dose immunoglobulin therapy, and thrombopoietin receptor agonists, as prior therapy for ITP, were 69.2, 38.5, and 26.9 %, respectively.

Primary efficacy endpoint

The percentage of patients who had achieved the platelet count ≥50 × 10^9/L at week 24 was 30.8 % (8/26 patients). The 95 % CI of the response rate was 14.3–51.8 %, and the lower limit of CI did not exceed the threshold of 20 %. However, in comparison with the response rate of 0–2 % in placebo group reported in other clinical studies with a similar target population to this study, it was suggested that the efficacy of rituximab observed in this study is substantial. The number of patients who achieved the platelet count ≥50 × 10^9/L at each time point for the assessment is shown in Table 2. Box plots of the trajectory of the platelet count over 24 weeks in a subgroup which consists of eight responders is shown in Fig. 1. In these eight responders, mean platelet count reached ≥50 × 10^9/L at week 4 and continued to increase throughout the follow-up period (Fig. 1).

Secondary efficacy endpoints

The percentage of patients with the platelet count ≥100 × 10^9/L and who did not have bleeding at week 24 was 15.4 % (4/26 patients; 95 % CI 4.4, 34.9 %). The percentage of patients with the platelet count ≥30 × 10^9/L and ≥twofold higher than the baseline value and who did not have bleeding at week 24 was 26.9 % (7/26 patients; 95 % CI 11.6, 47.8 %).

As for the percent changes of the platelet count, the median platelet count was significantly increased compared with the baseline at every time point from week 1 to week 24 ($P < 0.01$, signed rank sum test; Fig. 2). The median platelet count exceeded ≥30 × 10^9/L at week 8 and remained stable throughout the follow-up period.

The percentage of patients with Grade ≥1 hemorrhage in the WHO bleeding scale was numerically decreased at every time point compared with the baseline. Statistically significant decrease was observed at week 8 compared with the baseline (26.9 vs 57.7 %, $P = 0.0215$; exact

Table 2 Percentages of patients who had achieved the platelet count $\geq 50 \times 10^9$/L at each time point

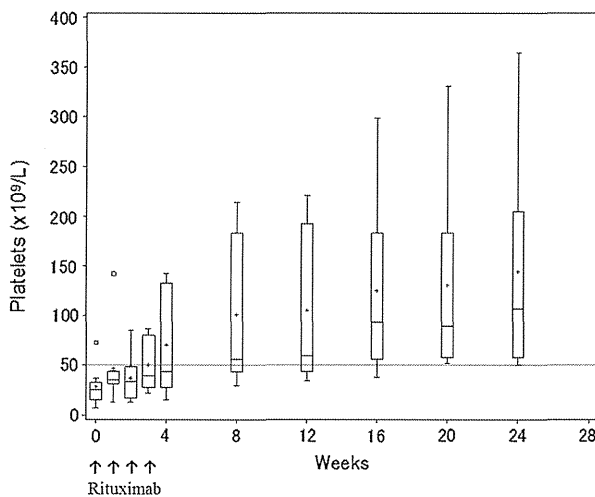| Time points	n	No. of patients who achieved	%	Two-sided 95 % CI of the percentage
Week 1	26	3	11.5	(2.4, 30.2)
Week 2	25	2	8.0	(1.0, 26.0)
Week 3	26	4	15.4	(4.4, 34.9)
Week 4	26	3	11.5	(2.4, 30.2)
Week 8	26	7	26.9	(11.6, 47.8)
Week 12	26	6	23.1	(9.0, 43.6)
Week 16	26	8	30.8	(14.3, 51.8)
Week 20	26	8	30.8	(14.3, 51.8)
Week 24	26	8	30.8	(14.3, 51.8)

Fig. 1 *Box plots* of platelet counts of the eight patients who met the primary response (platelet count >50 × 10^9/L at week 24). *Central horizontal bold line* is the median; the *lower* and *upper box* limits are the 1st and 3rd quartiles, respectively; and the *whiskers* extended to the most extreme data points, which do not exceed the 1.5 × the interquartile of the box. *Plus symbol* represents the mean value

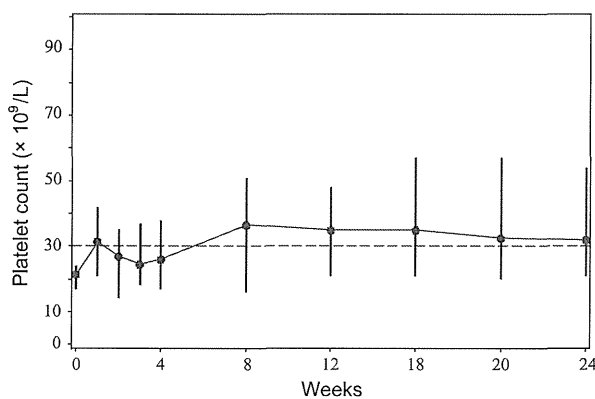**Fig. 2** Time course of median platelet count (FAS). *Black circles* are median values of platelet count. The *lower* and *upper ends* of *vertical lines* are the 1st and 3rd quartiles, respectively

McNemar's test), whereas no significant differences were observed at other time points.

Subgroup analysis

In Table 3, the results of subgroup analyses of the percentage of patients with the platelet count $\geq 50 \times 10^9$/L at week 24 were summarized. Subgroups with higher response rate were patients with duration of ITP shorter than the median duration 5.9 years (46.2 %, 6/13 patients), patients who underwent splenectomy (50.0 %, 2/4 patients), patients who did not have concomitants for ITP at baseline (60.0 %, 3/5 patients), patients who did not previously receive thrombopoietin receptor agonists (36.8 %, 7/19 patients), and patients with previous therapies for ITP less than 3 (41.2 %, 7/17 patients). While factors predictive of response to rituximab have not been consistently identified across studies, shorter duration of ITP was reported to be associated with good response from several studies [14, 15] as we found in this study.

Exploratory efficacy endpoints

Peripheral blood B cells (CD20-positive cells and CD19-positive cells) were significantly decreased at week 2 and subsequent time points compared with the baseline ($P < 0.001$ for both parameters: signed rank sum test). The median absolute B cell count at week 2 was <5 cells/μL and persisted in low during the study. Transient but significant decrease of peripheral blood T cells (CD3-positive cells) was observed at weeks 2, 4, and 12, compared with the baseline ($P < 0.05$, signed rank sum test); however, the median value of absolute cell count of CD3-positive T cells remain >790 cells/μL throughout the study.

Serum IgG levels were significantly increased at week 4 compared with the baseline ($P = 0.023$, signed rank sum test), whereas IgM levels were significantly decreased at weeks 12 and 24, compared with the baseline ($P < 0.001$, signed rank sum test). These changes of serum IgG and IgM, however, were within the normal range. Serum IgA