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BRIEF COMMUNICATION

Single nucleotide polymorphism of interleukin-1β associated with *Helicobacter pylori* infection in immune thrombocytopenic purpura

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Key words
Helicobacter pylori; immune thrombocytopenic purpura; infection; interleukin-1

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Abstract

To examine the role of genetic factors in development of immune thrombocytopenic purpura (ITP) in association with *Helicobacter pylori* infection, gene polymorphisms within the loci for human leukocyte antigen class II, interleukin (IL)-1β (−511), tumor necrosis factor-β (+252), immunoglobulin (Ig)G1 heavy chain (+643), and Igκ light chain (+573) were determined in 164 adults with ITP and 75 healthy controls. Of these gene polymorphisms, the IL-1β (−511) T allele was less frequently detected in *H. pylori*-infected than in *H. pylori*-uninfected (58% *vs* 81%, $P = 0.01$, odds ratio = 0.31) ITP patients diagnosed before age 50. These findings suggest that a single nucleotide polymorphism within the IL-1β (−511) may affect susceptibility to early-onset ITP associated with *H. pylori* infection.

Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by the presence of autoantibodies against platelet membrane glycoproteins, such as GPIIb/IIIa (1). Its pathogenic process primarily involves an accelerated clearance of opsonized platelets by phagocytes in the reticuloendothelial system (1). The etiology of ITP remains unclear, but both genetic and environmental factors are thought to play a role in the development of the disease. Several genes involved in immune system regulation, including human leukocyte antigen (HLA)-DPB1 and tumor necrosis factor (TNF)-β, are associated with susceptibility to ITP (2, 3). On the other hand, the potential association of ITP with infectious agents, such as HIV and hepatitis C virus, has also attracted many investigators (4). In addition, several recent lines of evidence have indicated that platelet recovery occurs in a subset of ITP patients infected with *Helicobacter pylori*, a gram-negative bacterium that establishes chronic infection in the gastric mucosa, after the successful eradication of *H. pylori* (5). Several potential mechanisms for the role of *H. pylori* infection in the ITP pathogenesis include molecular mimicry between *H. pylori* components and platelet antigens and modulation of host's immune system by *H. pylori* infection (6), but we have recently proposed a mechanism in which *H. pylori* modulates the Fcγ receptor balance of monocytes/macrophages toward inhibitory FcγRIIB, thereby enhancing phagocytosis and antigen presentation (7). In addition, chemokine production in response to *H. pylori* infection may contribute to the pathogenesis of ITP because serum levels of monocyte chemoattractant protein-1, regulated on

activation normally T-cell expressed and secreted, and epithelial cell-derived neutrophil attractant-78 were elevated in *H. pylori*-infected than in *H. pylori*-uninfected ITP patients (8). Interestingly, platelet recovery after *H. pylori* eradication was observed in nearly half of the patients in cohorts from Japan (9) and Italy (10), but in <15% of the patients in cohorts from Spain and the United States (11, 12). This apparent ethnic difference suggests a potential role for host genetic factors in the development of *H. pylori*-associated ITP. To investigate this issue, in this study, we examined polymorphisms of genes for selected immune system-regulating molecules, including TNF-β, interleukin (IL)-1β, immunoglobulin (Ig), and HLA class II in Japanese patients with ITP, with and without an associated *H. pylori* infection.

We studied 164 unrelated Japanese adult patients with chronic ITP, defined as thrombocytopenia (platelet count $<100 \times 10^9$/l) for at least 6 months, normal or increased bone marrow megakaryocytes, and no secondary diseases that could account for the thrombocytopenia (1). Refractory ITP was defined as a platelet count of $<50 \times 10^9$/l despite treatment with high-dose corticosteroids and splenectomy (1). The patients were enrolled consecutively and were followed at Keio University Hospital, Tokyo, Japan. None of the patients had ever been treated for *H. pylori* eradication. They included 54 men and 110 women with a mean age of 45.4 ± 17.0 years (range 17–80). Controls included 75 race-matched healthy volunteers living in the Tokyo metropolitan area. Written informed consent was obtained from all the participants in accordance with the Keio University Institutional Review Board guidelines.

Helicobacter pylori infection was defined by the presence of IgG anti-*H. pylori* antibody, which was measured in plasma samples using an enzyme-linked immunosorbent assay kit, HEL-pTEST (Amrad, Kew, Australia). The results showed that 85 patients (52%) were infected with *H. pylori*. Comparisons of the demographic and clinical characteristics were made between *H. pylori*-infected and *H. pylori*-uninfected ITP patients. Differences between two groups were evaluated for statistical significance using the Mann–Whitney *U*-test or 2 × 2 chi-square test as appropriate. The mean age at diagnosis was significantly higher for the *H. pylori*-infected than the *H. pylori*-uninfected patients (50.8 ± 16.0 *vs* 39.5 ± 16.3 years, $P = 0.002$). The female dominance was more prominent in the *H. pylori*-uninfected compared with *H. pylori*-infected patients (79% *vs* 57%, $P = 0.003$), but there was no difference in the frequency of refractory ITP, which was defined to be poor response to corticosteroids and splenectomy. The number of circulating B cells producing IgG autoantibodies to platelet GPIIb/IIIa measured using an enzyme-linked immunospot assay (13) were not different between the two groups.

We first compared the distribution of single nucleotide polymorphisms (SNPs) within TNF-β (+252 G/A), IL-1β (−511 C/T), IgG1 heavy chain (+643 G/A), and Igκ chain (+573 C/G) among *H. pylori*-infected and *H. pylori*-uninfected ITP patients and healthy controls. These SNPs were determined by the specific amplification of genomic DNA that contained individual SNPs by polymerase chain reaction (PCR), combined with separation on an agarose gel to detect restriction fragment length polymorphisms (RFLPs) or hybridization with sequence-specific oligonucleotide probes (3). The distribution of individual SNP phenotypes in each group conformed to the Hardy–Weinberg principle. Phenotypic frequencies were tested for statistical significance using the chi-square test. There was no significant difference in the distribution of these SNPs between *H. pylori*-infected and *H. pylori*-uninfected ITP patients, *H. pylori*-infected patients and healthy controls, or *H. pylori*-uninfected patients and healthy controls (Table 1). Our control samples are likely to be a representative for the Japanese population based on the similar allele and diplotype frequencies to the published data in terms of IL-1β (−511) SNP (14). The HLA-DRB1, -DQB1, and -DPB1 alleles were also determined using the PCR-RFLP method (15), but again there was no significant difference in the relative frequencies of individual alleles among *H. pylori*-infected and *H. pylori*-uninfected ITP patients and healthy controls (data not shown). In contrast, Veneri et al. reported that ITP patients with *H. pylori* infection show significantly higher frequencies of HLA-DRB1*11 and *14 and DQB1*03 and a significantly lower frequency of DRB1*03 than those without the infection (16). The reason for this inconsistent result is unknown, but it might be because of the difference in HLA class II allele distribution between the Italians and the Japanese: DRB1*03 is lacking in the Japanese population.

Because *H. pylori* infection is established during infancy and childhood and sustained throughout life (17), a long incubation period must be required for *H. pylori*-associated ITP to develop. Therefore, it is possible that the pathophysiologic process of ITP differs among *H. pylori*-infected patients according to the length of the incubation period. Based on this assumption, we divided the ITP patients into two groups according to their age at diagnosis: early-onset ITP (diagnosis before age 50) and late-onset ITP (diagnosis at or after age 50). When demographic and clinical characteristics were compared between these two groups (Table 2), females were significantly more common among the patients with early-onset ITP than those with late-onset ITP. *H. pylori* infection was significantly less frequent in the early-onset ITP *vs* late-onset ITP patients, indicating that the majority of the patients with *H. pylori* infection developed ITP more than 50 years after the original infection. As shown in Table 3, among patients with early-onset ITP, the distribution of IL-1β (−511) differed

Table 1 Phenotypic frequencies of single nucleotide polymorphisms within the genes for tumor necrosis factor (TNF)-β, interleukin (IL)-1β, immunoglobulin (Ig)G1 heavy chain, and Igκ chain in *Helicobacter pylori*-infected and *H. pylori*-uninfected immune thrombocytopenic purpura (ITP) patients and healthy controls

		ITP patients		
Gene location	Phenotype	*H. pylori* infected ($n = 85$), n (%)	*H. pylori* uninfected ($n = 79$), n (%)	Healthy controls ($n = 75$), n (%)
TNF-β (+252)	G/G	10 (12)	13 (16)	18 (24)
	G/A	42 (49)	36 (46)	27 (36)
	A/A	33 (39)	30 (38)	30 (40)
IL-1β (−511)	C/C	33 (39)	20 (25)	23 (31)
	C/T	30 (35)	39 (50)	35 (47)
	T/T	22 (26)	20 (25)	17 (22)
IgG1 heavy chain (+643)	G/G	1 (1)	1 (1)	1 (2)
	G/A	14 (17)	14 (18)	18 (28)
	A/A	68 (82)	64 (81)	45 (70)
Igκ chain (+573)	C/C	7 (8)	6 (8)	4 (7)
	C/G	36 (44)	31 (41)	30 (51)
	G/G	40 (48)	38 (51)	25 (42)

significantly between the *H. pylori*-infected and the *H. pylori*-uninfected individuals (corrected $P = 0.04$). The pairwise comparisons showed that IL-1β (−511) C/C was increased and C/T was decreased in patients with *H. pylori* infection compared with those without [$P = 0.01$, odds ratio (OR) = 3.2, 95% confidence interval (CI): 1.3–8.0 and $P = 0.02$, OR = 0.34, 95% CI 0.14–0.83, respectively]. Finally, the T allele at IL-1β (−511) was less common in the *H. pylori*-infected than in the *H. pylori*-uninfected patients (58% *vs* 81%, $P = 0.01$, OR = 0.31, 95% CI 0.13–0.77). There was no significant association between *H. pylori* infection and gene polymorphisms within other loci. In addition, in patients with late-onset ITP, there was no difference in distribution of any gene polymorphisms, including IL-1β (−511), between the presence and the absence of *H. pylori* infection.

This is the first report representing a potential association between SNPs within the IL-1β locus and the prevalence of *H. pylori* infection in patients with ITP. In particular, we found that a lack of the IL-1β (−511) T allele was associated with *H. pylori* infection in patients with early-onset ITP but not in those with late-onset ITP. IL-1β, a proinflammatory cytokine, is known to be upregulated in the *H. pylori*-infected gastric mucosa (18), suggesting that it is important in the pathogenic processes of *H. pylori*-associated gastric diseases, such as chronic gastritis and gastric cancer. IL-1β is not only involved in the inflammatory response at the gastric mucosa but also influences the level of gastric acid secretion. Namely, IL-1β inhibits the secretion of gastric acid by parietal cells, resulting in *H. pylori*'s distribution to the corpus (19). Although there are some conflicting data regarding the functional effect of the allelic dipolymorphism of IL-1β (−511) on the production of IL-1β, the T allele is associated with increased IL-1β production in patients with gastric cancer and in healthy controls (20, 21). The IL-1β (−511) T allele has nearly complete linkage disequilibrium with the IL-1β (−31) C allele, which amplifies the interaction between genomic DNA and transcriptional factors at the TATA-box and is associated with increased transcription efficiency (20). In fact, in *H. pylori*-infected patients with gastritis, the gastric juice pH is significantly higher in those with the IL-1β (−511) T allele than in those without (21). In addition, subjects

Table 2 Demographic and clinical characteristics of patients with early-onset and late-onset ITP[a]

Demographic and clinical findings	Early-onset ITP ($n = 97$)	Late-onset ITP ($n = 67$)	P value
Age at diagnosis (years)[b]	33.1 ± 9.0 (17–49)	63.1 ± 7.6 (50–80)	N/A
Female[c]	73%	58%	0.045
Refractory ITP[c]	8%	7%	0.8
Anti-GPIIb/IIIa antibody-producing B cells (per 10^5 PBMC)[b,d]	6.4 ± 4.5 (0.2–17.7)	6.6 ± 4.2 (1.4–18.9)	0.7
Helicobacter pylori infection[c]	39%	70%	<0.001

ITP, immune thrombocytopenic purpura; N/A, not applicable; PBMC, peripheral blood mononuclear cells.

[a] ITP patients were divided into two groups according to their age at diagnosis (<50 years as early onset and ≥50 years as late onset).

[b] Continuous variables are shown as the mean ± SD (range) and were statistically evaluated using the Mann–Whitney *U*-test.

[c] Categorized variables were evaluated for statistical significance using the 2 × 2 chi-square test.

[d] This assay was performed in 57 patients with early-onset ITP and 44 patients with late-onset ITP.

Table 3 Phenotypic frequencies of single nucleotide polymorphisms within the genes for tumor necrosis factor (TNF)-β, interleukin (IL)-1β, immunoglobulin (Ig)G1 heavy chain, and Igκ chain in early-onset immune thrombocytopenic purpura patients with and without *Helicobacter pylori* infection

Gene location	Phenotype	*H. pylori* infected ($n = 38$), n (%)	*H. pylori*-uninfected ($n = 59$), n (%)	Overall corrected P value[a]
TNF-β (+252)	G/G	4 (11)	11 (19)	0.7
	G/A	19 (50)	28 (47)	
	A/A	15 (39)	20 (34)	
IL-1β (−511)	C/C	16 (42)	11 (19)	0.04
	C/T	10 (26)	30 (51)	
	T/T	12 (32)	18 (30)	
IgG1 heavy chain (+643)	G/G	1 (3)	1 (2)	0.7
	G/A	4 (11)	11 (19)	
	A/A	32 (86)	47 (79)	
Igκ chain (+573)	C/C	4 (11)	6 (10)	1
	C/G	18 (47)	26 (46)	
	G/G	16 (42)	25 (44)	

[a] Phenotypic frequencies were tested for statistical significance using the 2 × 3 chi-square test. Corrected P value was obtained by multiplying the observed P values by the number of comparisons made.

carrying the T allele are reported to have an increased risk of developing gastric atrophy and cancer (20–22). This association is thought to be because of both the more extensive gastritis and the lower acid production resulting from the high IL-1β production in subjects carrying the T allele, who would increase the exposure of gastric epithelium to exogenous and endogenous stimulants.

If this is the case, our results suggest that individuals lacking the T allele, who are infected with *H. pylori* and thus have less extensive gastritis and high acid production, have an increased risk for developing ITP before age 50. It is likely that the *H. pylori* strains that colonize the stomach are different between subjects with and without the T allele because the intragastric environment is apparently different. In this regard, a recent report showed the eradication rates for *H. pylori* by a standard triple regimen to be 77%, 90%, and 95% in subjects with the C/C, C/T, and T/T phenotypes of IL-1β (−511), respectively (23), suggesting that the IL-1β (−511) SNP could be a prognostic indicator of the success or failure of *H. pylori* eradication. Moreover, the frequency of *H. pylori* strains possessing the *cagA* gene is significantly higher in ITP patients than in healthy controls (24). These particular strains may induce ITP in infected individuals within a shorter incubation period compared with other *H. pylori* strains. Interestingly, IL-1β (−511) T carriers infected with *cagA*-positive strains were found to have an increased risk for developing gastric carcinoma (22). Taken together, a combination of host genetic factors and infectious agents may be important for determining an individual's susceptibility to ITP in the setting of *H. pylori* infection. Further studies investigating the interactive effects of host genetic factors and colonized *H. pylori* strains on the development of ITP are necessary to elucidate the role of *H. pylori* in the pathogenesis of ITP.

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Heterogeneous pathogenic processes of thrombotic microangiopathies in patients with connective tissue diseases

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Summary

To clarify the pathogenic processes of thrombotic microangiopathies (TMAs) in patients with connective tissue disease (CTD), we analysed clinical characteristics and plasma ADAMTS13 levels in 127 patients with CTD-TMAs, including patients with systemic lupus erythematosus (SLE), systemic sclerosis, polymyositis/dermatomyositis, and rheumatoid arthritis (RA), and 64 patients with acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP). Plasma levels of ADAMTS13 activity, antigen, and inhibitors were determined by enzyme immunoassays. IgG type anti-ADAMTS13 antibodies were also detected by immunoblots using purified ADAMTS13. ADAMTS13 activity was significantly decreased in CTD-TMAs, regardless of the underlying disease, but the frequency of severe deficiency (defined as <0.5% of normal) was lower in CTD-TMA patients than in ai-TTP patients (16.5% vs. 70.3%, $p<0.01$). Severe deficiency of ADAMTS13 activity was predominantly detected in patients with RA- and SLE-TMAs, and was closely associated with the presence of anti-ADAMTS13 IgG antibodies. CTD-TMA patients with severe deficiency of ADAMTS13 activity appeared to have lower platelet counts and better therapeutic outcomes. At least two phenotypic TMAs occur in patients with CTDs: a minor population with deficient ADAMTS13 activity caused by neutralising autoantibodies, and a major population with normal or moderately reduced activity. Classifying CTD-TMAs by ADAMTS13 activity may be useful in predicting the clinical course and therapeutic outcomes, as patients with moderately reduced activity are likely to have more prominent renal impairment and poor prognoses.

Keywords

Connective tissue disease, TMA, ADAMTS13, autoantibody

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Introduction

Thrombotic thrombocytopenic purpura (TTP) and haemolytic uraemic syndrome (HUS) are life-threatening diseases, characterised pathologically by thrombotic microangiopathies (TMAs), microangiopathic haemolytic anaemia, destructive thrombocytopenia, and organ dysfunction caused by platelet-thombi (1). The discovery of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motif 13) (2–7), which specifically cleaves the Tyr1605-Met1606 bond in the von Willebrand factor (VWF)-A2 domain, facilitated the recognition that enzymatic deficiency due to genetic mutation or acquired autoantibodies was a more specific feature of TTP (8, 9). In the absence of ADAMTS13, unusually large VWF multimers (UL-VWFMs) are not appropriately cleaved in circulation; as a result, platelets aggregate excessively under high shear stress (10, 11).

In 1939, Gitlow and Goldmark (12) first reported a close relationship between TTP and systemic lupus erythematosus (SLE). Since then, this concept has become well accepted (13–16), but investigations of the underlying pathogenic processes are still lacking. More recently, several investigators (17–21) have observed deficient ADAMSTS13 activity caused by inhibitory IgG-autoantibodies in some patients with SLE-related TTP; however, many other patients have slightly reduced or almost normal activity (18). Thus, the pathogenesis of SLE-related TTP is still controversial.

During 1997–2006, we identified a total of 783 patients with TMAs by analysing their clinical and laboratory findings, including the levels of ADAMTS13 activity (ADAMTS13:AC)

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and ADAMTS13 inhibitor (ADAMTS13:INH), at Nara Medical University (22). In this database, we found 33 patients with congenital ADAMTS13:AC deficiency (termed Upshaw-Schulman syndrome), a rare thrombo-haemorrhagic disease, and elucidated their ADAMTS13 genetic status (23, 24). Using the same database, we identified 127 patients who developed TMAs in association with connective tissue diseases (CTDs), including SLE, systemic sclerosis (SSc), polymyositis/dermatomyositis (PM/DM), and rheumatoid arthritis (RA).

In this study, to further characterise the clinical expression and underlying pathogenesis of CTD-TMAs, we have extensively evaluated ADAMTS13 profiles, antibodies to ADAMTS13, and clinical features in patients with CTD-TMAs in comparison with 64 patients with acquired idiopathic (ai)-TTP.

Patients, materials and methods

Study subjects

One hundred twenty-seven patients with CTD-TMAs in a Nara Medical University database (22) included 64 SLE patients, 42 SSc patients, 11 PM/DM patients, and 10 RA patients. All patients fulfilled the corresponding classification criteria (25–28). SSc patients with complicating classical hypertensive renal crisis were excluded from the analysis. Blood samples and detailed clinical information were provided by referring physicians in area hospitals across Japan, and only patients who were confirmed to have TMAs were included in the database.

Based on the previous reports (29–31), TMAs were defined as having all of the followings: (i) microangiopathic haemolytic anemia (haemoglobin ≤12 g/dl), Coombs test negative, undetectable serum haptoglobin (<10 mg/dl), more than two fragmented red cells (schistocytes) in a microscopic field with a magnification of 100, and concurrent increased serum lactate dehydrogenase (LDH) above institutional baseline; (ii) thrombocytopenia (platelet count ≤100 x 10^9/l); and (iii) a variety of organ dysfunction (renal or neurological involvement) devoid of the stigmata of disseminated intravascular coagulation.

Sixty-four ai-TTP patients in a Nara Medical University database generated during the past two years (March 2006-April 2008) were used in this study for comparison. A diagnosis of ai-TTP was made for patients (i) without apparent underlying disease, and (ii) with the aforementioned clinical and laboratory features of TMAs.

Normal plasma samples were prepared from 20 healthy individuals (10 male, 10 female) between 20 and 40 years of age for use as a control. The study protocol conformed to the ethical principles of the World Medical Association Declaration of Helsinki as reflected in a *priori* approval from the Ethics Committee of Nara Medical University, and written informed consent was obtained from all patients at each referral hospital.

Therapeutic regimens and outcomes

Steroid pulse therapy is administration of intravenous methylprednisolone 1 g/day for 3 days, followed by a moderate or high dose of steroid therapy (0.5–1.0 mg of oral prednisolone/kg body weight/day). Therapeutic plasma exchange was conducted according to the following regimen: daily plasma exchange (PE) was performed at 1.5-fold body plasma volume with fresh frozen plasma (FFP) for the first 3 days, and PE was then performed at one body plasma volume daily for up to 14 days, until normal platelet counts (>150 x 10^9/l) were achieved. Response to therapy for TMAs was evaluated in two ways: (i) remission, defined as normalisation of platelet count (>150 x 10^9/l) and clinical manifestations without requiring PE, or (ii) death due to TMA.

Blood sampling

Before therapeutic plasma exchange or plasma infusions, blood samples (4.5 ml) were taken from each patient into plastic tubes containing 0.5 ml of 3.8% sodium citrate. Plasma was isolated by centrifugation at 3,000 x g for 15 minutes (min) at 4°C. Plasma samples were kept in aliquots at –80°C until testing, and sent to our institution.

Plasma ADAMTS13:AC, ADAMTS13:INH, ADAMTS13:AG, and VWF:Ag

Plasma levels of ADAMTS13:AC were determined using a commercially available chromogenic act-ELISA (Kainos Inc., Tokyo, Japan) (32). The detection limit of ADAMTS13:AC by this method was 0.5% of the control. A good correlation in plasma levels of ADAMTS13:AC between the classic VWFM assay and the chromogenic ADAMTS13-act-ELISA has previously been shown in normal individuals (R^2=0.72, $p<0.01$) (32, 33).

In terms of plasma levels of ADAMTS13:AC, since SSC-ISTH (Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis) has not defined "severe" deficiency of ADAMTS13:AC, we have here tentatively categorized into three types of plasma ADAMTS13:AC: less than 0.5%, 0.5% to less than 25%, and 25% or higher of the normal control as severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal, respectively.

ADAMTS13:INH titers were evaluated by act-ELISA using plasma that was heat-inactivated at 56°C for 30 min. Inhibitor titers are expressed as Bethesda units (BU) (34). One BU is defined as the amount necessary to reduce ADAMTS13:AC to 50% of control levels. Titers greater than 0.5 Bethesda U/ml were classified as inhibitor-positive.

Plasma ADAMTS13 antigen (ADAMTS13:AG) levels were analysed by sandwich antigen (ag)-ELISA, using two murine anti-ADAMTS13 mAbs, A10 and C7. The A10 antibody recognizes an epitope in the disintegrin-like domain, completely inhibiting enzyme activity at a final concentration of 10 μg/ml (35). The C7 antibody recognizes the 7th and 8th thrombospondin-1 domains without affecting activity. The detection limit of the ag-ELISA for plasma ADAMTS13:AG was 0.1% of the normal control (35).

Plasma levels of VWF:Ag were assayed by sandwich ELISA using rabbit anti-human VWF polyclonal antibodies (DAKO, Denmark). The detection limit of this assay was 0.3% of the normal control.

Detection of IgG autoantibodies specific to ADAMTS13

Plasma-derived ADAMTS13 was purified using A10-agarose immunoaffinity chromatography followed by size-exclusion chromatography. Purified ADAMTS13 had a specific activity of 302 units/mg. SDS-5% polyacrylamide gel electrophoresis (PAGE) analysis revealed a 170kD-band before and a 190 kD-band after reduction (36).

To detect IgG-type autoantibodies specific for ADAMTS13, 0.15 μg purified ADAMTS13 per lane was separated by SDS-5% PAGE under non-reducing conditions, and electrophoretically transferred to polyvinylidine fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). After blocking nonspecific binding with 5% skim milk, PVDF membranes were cut longitudinally into small pieces (3 x 800 mm). Each strip was incubated overnight at 4°C with 3 ml 5% skim milk containing 50 μl heat-treated plasma from each CTD-TMA patient. The heat-treated plasma was prepared by incubation at 56°C for 30 min. After centrifugation, the supernatant was used in assays. Human IgG bound to the purified ADAMTS13 on PVDF membranes was detected using a horseradish peroxidase (HRP)-conjugated anti-human IgG polyclonal antibody (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA). Binding was visualised using the Western Lightning Chemiluminescence reagent (Perkin-Elmer Life Science Inc., Boston, MA, USA) and imaged by X-ray autoradiography (Eastman Kodak, Rochester, NY, USA) (37, 38). Heated plasma from a patient with acquired idiopathic TTP with IgG inhibitors against ADAMTS13 was used as a positive control, while that from a normal individual without ADAMTS13:INH was used as a negative control.

Statistical analysis

All continuous values are shown as median values (25, 75 percentile). All comparisons among three (severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal ADAMTS13 activity in both CTD-TMAs and ai-TTP) or five (SLE, SSc, PM/DM, RA, and ai-TTP) patient groups were tested for statistical significance using the Kruskal-Wallis H test or chi-square tests with Yates' correction for 2 x3 or 2 x 5 tables. Significant differences between three or five groups (overall $p < 0.05$) were further analysed by the Mann-Whiteney U-test or chi-square test. A two-tailed p-value less than 0.05 was considered to be significant.

Results

Clinical and laboratory features of CTD-TMAs

The clinical features and therapeutic outcomes of 127 patients with CTD-TMAs in comparison to 64 patients with ai-TTP are summarised in Table 1. SLE patients were younger at age at onset than patients with PM/DM or RA. There was a gender disparity, with female predominance, for patients with CTD-TMAs relative to ai-TTP patients. Platelet counts in SSc-TMA patients appeared to be higher than in patients with other CTD-TMAs or

Table 1: Clinical features and therapeutic outcomes of patients with CTD-TMAs and ai-TTP.

	CTD-TMAs (n=127)				ai-TTP (n=64)	Overall *P* *
	SLE (n=64)	SSc (n=42)	PM/DM (n=11)	RA (n=10)		
Clinical features						
Median age at onset of TMAs, years (25, 75 percentile)	44 (30, 54)	59 (54, 70)	57 (49, 63)	62 (56, 73)	54 (40, 69)	<0.01[a]
Female (%)	84	95	82	90	64	<0.01[b]
Renal involvement (%)	91	95	100	100	83	NS
CNS involvement (%)	69	48	64	80	70	NS
Laboratory findings at TMA diagnosis						
Median platelet count, 10^9/l (25, 75 percentile)	29 (9, 40)	50 (31, 74)	32 (9, 46)	23 (14, 28)	9 (9, 20)	NS
Median haemoglobin, g/dl (25, 75 percentile)	7.5 (6.1, 8.8)	8.3 (7.3, 9.3)	7.4 (6.6, 9.0)	7.2 (6.9, 8.1)	8.1 (6.4, 9.2)	NS
Median serum creatinine, mg/dl (25, 75 percentile)	1.6 (0.7, 2.6)	2.8 (1.9, 3.3)	1.5 (1.2, 2.3)	3.1 (1.1, 4.4)	2.1 (0.7, 2.1)	<0.01[c]
Median VWF:Ag , % (25, 75 percentile)	207 (147, 325)	256 (191, 370)	339 (225, 461)	302 (245, 454)	147 (114, 202)	<0.01[d]
Therapies						
Plasma exchange (%)	70	79	81	60	77	NS
Plasma infusion without plasma exchange (%)	27	21	18	40	25	NS
Steroid therapy without pulse therapy (%)	53	40	82	60	39	NS
Steroid pulse therapy (%)	38	26	0	20	30	NS
Rituximab (%)	0	0	0	0	9	NS
Immunosuppressants (%)	31	14	9	20	10	NS
Therapeutic response	(n=50)	(n=26)	(n=7)	(n=9)	(n=61)	
Remission of TMAs (%)	74	42	57	33	79	<0.01[e]
Death due to TMAs (%)	26	58	43	67	21	<0.01[f]

NS: not significant differences ($P \geq 0.05$). Overall *P* values were calculated using the Kruskal-Wallis H test or chi-square tests with Yates' correction for 2 x 5 tables. Significant differences between 5 groups (overall $P < 0.5$) were further analyzed by Mann-Whiteney U-test or chi-square test. [a] $P<0.01$ between SLE and PM/DM, RA. [b] $P<0.01$ between SLE, SSc, PM/DM, RA and ai-TTP. [c] $P<0.05$ between SLE and RA. [d] $P<0.01$ between SLE, and PM/DM. $P<0.01$ between PM/DM, RA and ai-TTP. [e] $P<0.01$ between SLE and SSc. $P<0.01$ between SSc, RA and ai-TTP. $P<0.05$ between SLE and RA. [f] $P<0.01$ between SLE and SSc. $P<0.01$ between SSc, RA and ai-TTP. $P<0.05$ between SLE and RA.

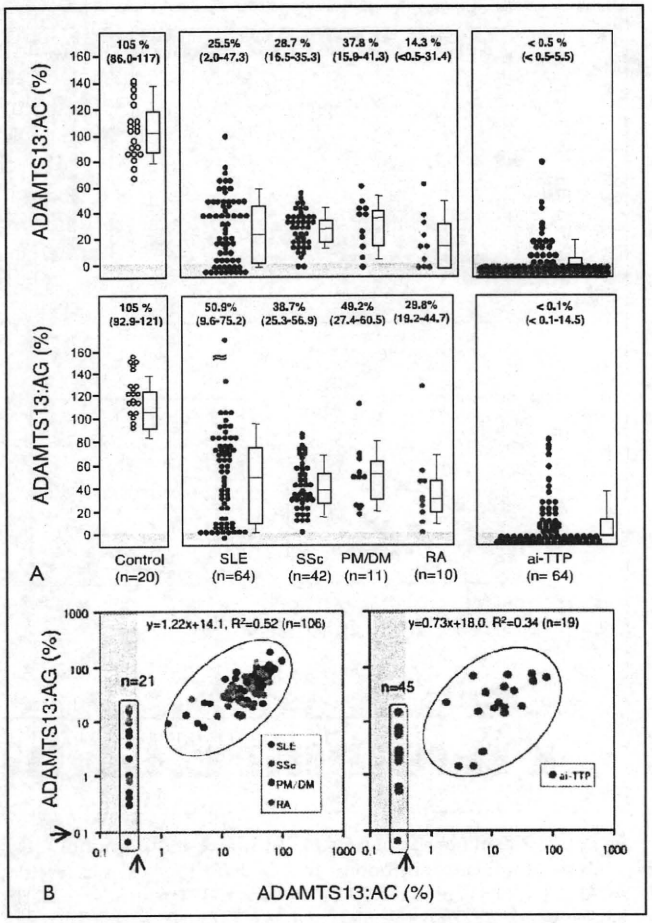

Figure 1: Plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with CTD-TMA or ai-TTP. A; top) Plasma levels of ADAMTS13:AC measured by chromogenic ADAMTS13-act-ELISA in patients with CTD-TMA or ai-TTP. The median values (25, 75 percentiles) of ADAMTS13:AC in these patients are shown at the top of the figure. A; bottom) Plasma levels of ADAMTS13:AG measured by ag-ELISA. The median values (25, 75 percentiles) of ADAMTS13:AG in these patients are shown at the top of the figure. B) Relationship between plasma levels of ADAMTS13:AC (x) and ADAMTS13:AG (y).

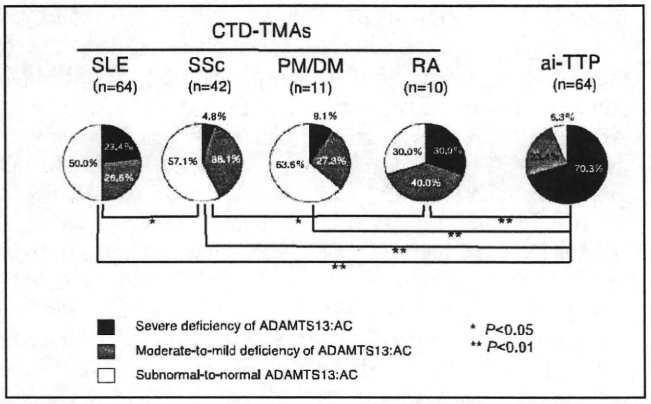

Figure 2: The proportion of CTD-TMA or ai-TTP patients grouped by three levels of plasma ADAMTS13:AC. The proportion of severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal plasma ADAMTS13:AC is illustrated in this figure. Comparison among five patient groups was tested for statistical significance using chi-square tests with Yates' correction for 3 x 5 tables. Significant differences between five groups (overall $p < 0.01$) were found. Comparison between pairs of groups was further investigated by chi-square test.

ai-TTP. Serum creatinine levels of patients with SLE were significantly lower in patients with RA. Interestingly, the plasma levels of VWF:Ag in patients with PM/DM and RA were significantly higher than in ai-TTP patients. PE was conducted in the majority of patients in all groups. This therapeutic approach resulted in a high remission rate in patients with SLE and ai-TTP, but was less effective in patients with SSc and RA.

Plasma levels of ADAMTS13:AC and ADAMTS13:AG

The average plasma levels of ADAMTS13:AC in each category of CTD-TMA and ai-TTP were significantly decreased compared to the levels in normal controls ($p<0.01$) (Fig. 1A top). On the other hand, plasma levels of ADAMTS13:AC in patients with CTD-TMAs were increased compared to the levels in ai-TTP patients. The distribution of ADAMTS13:AC was quite different depending on the category of CTD (Fig. 2). The proportion of severe ADAMTS13:AC deficiency in SSc patients was lower than that in SLE, PM/DM, and RA patients. In contrast, 70.3% of the 64 patients with ai-TTP had severe ADAMTS13:AC deficiency, higher than that of any individual category of CTD-TMA ($p<0.01$).

Plasma levels of ADAMTS13:AG in each category of CTD-TMA patients were also significantly decreased relative to the levels in normal controls ($p<0.01$). Further, plasma levels of ADAMTS13:AG in patients with CTD-TMA were also increased relative to the levels in ai-TTP patients (Fig. 1A bottom).

Next, we examined the relationship between the plasma levels of ADAMTS13:AC and ADAMTS13:AG in both the CTD-TMA and ai-TTP patient groups (Fig. 1B). Twenty-one CTD-TMA patients with severe deficiency ADAMTS13:AC had ADAMTS13:AG levels ranging from less than 0.1% to 19.1% of normal control. On the other hand, 106 patients with detectable ADAMTS13:AC (≥0.5%) had a good correlation between plasma levels of ADAMTS13:AC and ADAMTS13:AG levels ($R^2=0.52$, $p<0.01$). However, the plasma levels of ADAMTS13:AG were almost always higher than the levels of ADAMTS13:AC (Fig. 1B left). This is probably because the presence of circulating anti-ADAMTS13 antibody and ADAMTS13 antigen complexes. These observations were comparable to ai-TTP, in which 45 patients with undetectable ADAMTS13:AC had ADAMTS13:AG levels ranging from less than 0.1% to 14.8% of normal control, whereas 19 patients with detectable ADAMTS13:AC had a good correlation between plasma levels of ADAMTS13:AC and levels of ADAMTS13:AG ($R^2=0.34$, $p<0.01$) (Fig. 1B right).

Severe deficiency of ADAMTS13:AC associated with autoantibodies to ADAMTS13

To investigate the role of anti-ADAMTS13 autoantibodies in severe deficiency of ADAMTS13:AC, we evaluated plasma levels of ADAMTS13:INH in the CTD-TMA and ai-TTP patient groups (Fig. 3A). In 65 of 127 (51.2%) CTD-TMA patients,

plasma levels of ADAMTS13:INH were greater than 0.5 BU/ml. Interestingly, a significant variation was observed, depending on the underlying disease: 60% of RA patients, 54.5% of PM/DM patients, 50% of SLE patients, and 50% of SSc patients had ADAMTS13:INH levels greater than 0.5 BU/ml. In contrast, 55 (85.9%) ai-TTP patients had plasma levels of ADAMTS13:INH greater than 0.5 BU/ml.

Twenty-one CTD-TMA patients with undetectable ADAMTS13:AC had ADAMTS13:INH levels ranging from 0.7 to 125 BU/ml. However, the patients with detectable ADAMTS13:AC had ADAMTS13:INH levels between <0.5 and 1.0 BU/ml, and no correlation between these two parameters was found (R^2=0.021) (Fig. 3B left). Similarly, in ai-TTP patients with undetectable ADAMTS13:AC, ADAMTS13:INH levels ranged from 0.5 to 20 BU/ml, and those patients with detectable ADAMTS13:AC had ADAMTS13:INH levels between <0.5 and 1.5 BU/ml. No correlation between these two parameters was found (R^2=0.058) for this patient group (Fig. 3B right).

As shown in Figure 3C, 21 CTD-TMA patients with undetectable ADAMTS13:AC were analysed for IgG-autoantibodies to ADAMTS13 by immunoblotting. A total of 18 of 21 (86%) patient plasmas displayed a 170 kD-band, indicating the presence of IgG-autoantibodies reacting with the purified ADAMTS13 under non-reducing conditions.

Plasmas from CTD-TMA patient groups with detectable ADAMTS13:AC were analysed by immunoblotting, and positive IgG-autoantibodies were detected in only 2/40 (5%) patients with moderate-to-mild ADAMTS13:AC deficiency, and 0/66 (0%) patients with subnormal-to-normal activity (data not shown).

Thus, in CTD-TMA patients with severe deficiency of ADAMTS13:AC, the presence of ADAMTS13:INH apparently had a high correlation with the appearance of IgG-autoantibodies reacting with purified ADAMTS13.

Clinical features and therapeutic outcomes of patients evaluated by plasma levels of ADAMTS13:AC

The clinical features and laboratory findings as well as therapy and outcome in the CTD-TMA and ai-TTP patient groups have been evaluated and categorised relative to three plasma levels of ADAMTS13:AC: severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal activity (Table 2). In CTD-TMA patients, gender disparity (female predominance) was not remarkable among the three groups of CTD-TMA patients. The frequency of renal involvement was apparently lower in patients with severe deficiency than in other CTD-TMA patients (p<0.01). Both platelet counts and serum creatinine levels of patients with severe ADAMTS13:AC deficiency tended to be lower than those in patients with moderate-to-mild deficiency or subnormal-to-normal activity. Plasma levels of VWF:Ag were in almost the same ranges in all three groups. These clinical features associated with the levels of ADAMTS13:AC were also observed in ai-TTP patients. But, there was no apparent difference in response to individual therapeutic regimens. Further, the tendency of higher remission and lower mortality rates was observed in patients with severe deficiency than in those with moderate-to-mild deficiency and with subnormal–to-normal activity (p=0.053).

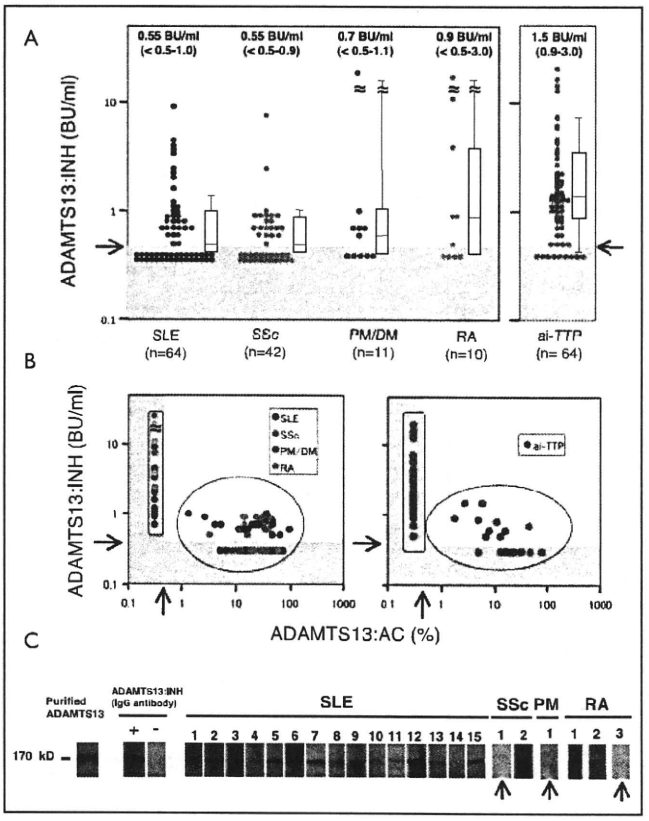

Figure 3: Plasma levels of ADAMTS13:INH and immunoblot analysis of IgG-autoantibodies to ADAMTS13. A) Plasma levels of ADAMTS13:INH in both the CTD-TMA and ai-TTP patient groups. The median values (25, 75 percentiles) of ADAMTS13:INH in these patients are shown at the top of the figure. B) Relationship between plasma levels of ADAMTS13:AC (x) and ADAMTS13:INH (y). C) Detection of IgG-autoantibodies specific to purified ADAMTS13 by immunoblot under non-reducing conditions. A total of 18 of 21 (86%) patient plasmas displayed a 170 kD-band, indicating the presence of IgG-autoantibodies reacting with purified ADAMTS13. The samples with an arrow indicate the IgG-autoantibodies negative (see *Results* for detail). In the left lane, SDS-5% PAGE analysis of purified pd-ADAMTS13 revealed a 170kD-band before reduction. In the next lane, heated plasma from ai-TTP patient with IgG inhibitors against ADAMTS13 (+) displayed a 170kD-band as a positive control, while that from normal individual without ADAMTS13:INH (-) showed no band (a negative control).

Discussion

By analysing 127 CTD-TMA patients, we have shown heterogeneous pathogenic processes of the TMAs, with a minor population defined by severe deficiency of ADAMTS13:AC due to the presence of IgG-inhibitors, and a major population consisting of two subgroups categorised by plasma levels of ADAMTS13:AC: moderate-to-mild deficiency with or without inhibitors, and subnormal-to-normal activity without inhibitors. The percentage of severe deficiency of ADAMTS13:AC in each patient group showed significant variation depending on the underlying disease: severe deficiency was more frequent in patients associated with RA or SLE, and was much less frequent in patients with SSc or PM/DM. These results contrast sharply with

64 ai-TTP patients, which showed a major population (70.3%) with severe deficiency of ADAMTS13:AC due to IgG-inhibitors, and two minor populations of 23.4% with moderate-to-mild deficiency and 6.3% with subnormal-to-normal activity.

Among the clinical features of CTD-TMA patients prior to treatment, serum creatinine levels and platelet counts appeared to be lower in the group with severe deficiency of ADAMTS13:AC than in the moderate-to-mild and subnormal-to-normal subgroups. However, the levels of haemoglobin and plasma VWF:Ag were almost indistinguishable among these three patient groups. Similarly, for ai-TTP patients, the levels of serum creatinine in the group with severe deficiency of ADAMTS13:AC tended to be lower than those of the other two subgroups. These results were in agreement with a previous report of ai-TTP by Vesely et al. (39).

CTD-TMA patients with severe deficiency of ADAMTS13:AC seemed to have better outcomes than patients with moderate-to-mild deficiency (p=0.078). Similarly, the therapeutic outcomes of ai-TTP patients were also likely to be better in patients with severe deficiency of ADAMTS13:AC than in those with moderate-to-mild deficiency (p=0.094), as previously reported (39–41). Thus, except for the differences in proportion of the CTD-TMA with severe ADAMTS13:AC deficiency (16.5%) and ai-TTP with severe deficiency (70.3%), the clinical features and therapeutic outcomes were comparable in these two patient groups.

Pathogenesis of CTD-TMA with severe deficiency of ADAMTS13:AC due to IgG-autoantibodies can be explained in a manner similar to typical ai-TTP: in the absence of ADAMTS13:AC the UL-VWFMs produced in vascular en-

Table 2: Clinical features and therapeutic outcomes according to the levels of plasma ADAMTS13:AC.

	CTD-TMAs (n=127)			ai-TTP (n=64)		
	Severe deficiency (n=21)	**Moderate-to-mild deficiency (n=40)**	**Subnormal-to-normal activity (n=66)**	**Severe @deficiency (n=45)**	**Moderate-to-mild deficiency (n=15)**	**Subnormal-to-normal activity (n=4)**
ADAMTS13:AC (%)	< 0.5	0.5 ~ <25	25 ~	< 0.5	0.5 ~ <25	25 ~
Clinical features						
Median age at onset of TMAs, years (25, 75 percentile)	44 (30, 56)	60 (54, 68)	49 (22, 55)	46 (37, 62)	69 (54, 75)	77 (74, 82)
Female (%)	86	90	88	71	53	25
Renal involvement (%)	71*	100*	97*	78	93	100
CNS involvement (%)	71	73	53	67	87	50
Laboratory findings at TMA diagnosis						
Median platelet count, 10^9/l (25, 75 percentile)	8 (6, 14)	37 (20, 55)	41 (22, 55)	15 (8, 16)	19 (10, 24)	19 (8, 47)
Median haemoglobin, g/dl (25, 75 percentile)	7.6 (6.8, 9.2)	7.3 (6.6, 8.0)	7.8 (6.4, 9.2)	7.8 (6.1, 9.0)	8.1 (7.2, 9.8)	7.5 (6.1, 10.3)
Median serum creatinine, mg/dl (25, 75 percentile)	0.9 (0.6, 1.6)	2.7 (1.3, 4.2)	2.3 (1.2, 3.1)	1.2 (0.7, 1.2)	2.2 (1.4, 3.0)	5.0 (3.6, 11.3)
Median VWF:Ag , % (25, 75 percentile)	207 (169, 316)	248 (190, 362)	273 (164, 380)	143 (115, 199)	147 (104, 213)	171 (119, 248)
Therapies						
Plasma exchange (%)	81	70	73	82	53	100
Plasma infusion without plasma exchange (%)	24	23	27	15	40	75
Steroid therapy without pulse therapy (%)	100	80	76	60	60	50
Steroid pulse therapy (%)	100	80	76	11	20	25
Rituximab (%)	0	0	0	11	0	0
Immunosuppressants (%)	33	18	23	7	13	0
Therapeutic response	(n=21)	(n=31)	(n=41)	(n=45)	(n=13)	(n=3)
Remission of TMAs (%)	76	45	61	87	54	67
Death due to TMAs (%)	24	55	39	13	46	33

All comparisons among three groups (severe deficiency, moderate-to-mild deficiency, and subnormal-to-normal ADAMTS13 activity in both CTD-TMAs and ai-TTP) were tested for statistical significance using the Kruskal-Wallis H test or chi-square tests with Yates' correction for 2 x 3 tables. Significant differences between 3 groups (overall $P < 0.05$) were further analyzed by Mann-Whiteney U-test or chi-square test. *Significant difference ($P<0.05$) was only found in renal involvement between severe deficiency and moderate-to-mild deficiency, subnormal-to-normal activity of CTD-TMAs ($P<0.01$).

dothelial cells and released into circulation aggregate platelets excessively under high shear stress, which is exclusively generated at microvasculature networks with luminal narrowing, hyperviscosity, and high flow rate of blood (10, 42).

On the other hand, for the patient subgroups of CTD-TMA with moderate-to-mild deficiency and subnormal-to-normal ADAMTS13:AC, which represent the major population here, it is hard to address the underlying mechanisms. It is well known that congenital deficiency of ADAMTS13:AC (Upshaw-Schulman syndrome) may have mild or absent clinical signs during childhood (23, 24, 43). However, once a rapid increase of plasma VWF, mediated by inflammation, pregnancy, or DDAVP (1-deamino-8-D-arginine vasopressin) administration occurs, TMA bouts are consistently induced (23, 24, 43). These observations indicate that the appearance of TMA bouts depends on the equilibrium between the amount of enzyme (ADAMTS13) and substrate (UL-VWFMs), and may be induced by an extremely low enzyme-to-substrate ratio in circulation (37, 44). In fact, the frequency of TMA has been reported to be 1–6% in the CTD patient population (45), whereas the annual incidence of ai-TTP-HUS has been reported to be 0.00037%-0.00 0446% (3.7–4.46 per million) per year (46, 47). We assume that a high prevalence of TMA bouts associated with CTD might be closely related to high VWF plasma levels (207–339%), together with deficient or moderate-to-mild decrease of plasma ADAMTS13:AC (Table 2). Anatomical changes of the microvasculature in CTD-TMA patients are another point of interest. These patients have narrowed vessel cavities due to the proliferation of vascular endothelial cells, and this generates higher shear stress. Under these circumstances, platelets tend to aggregate more extensively and elevated plasma levels of VWF with or without UL-VWFMs accelerate this reaction, causing platelet thrombi to form. Impaired vascular endothelial function due to vasculitis caused by CTDs may also prevent the efficient cleavage of UL-VWFMs by ADAMTS13. This speculation is derived from recent observations, in which ADAMTS13 most efficiently works as a solid-phase enzyme, after binding to cell surfaces such as vascular endothelial cells and platelets via hitherto unrecognised anchoring systems (48). We therefore speculate that anatomical changes such as luminal narrowing of blood vessel walls alter haemodynamics and generate high shear stress, and the impaired vessel walls fail to bind ADAMTS13 for the efficient cleavage of UL-VWFMs. Coexistence of these two factors apparently accelerates platelet clumping in the presence of high plasma levels of VWF, leading to TMA. High prevalence of CTD-TMA, despite lower frequency of severe deficiency of ADAMTS13:AC, may be explained by these mechanisms.

Since this study was conducted at Nara Medical University on behalf of patients and physicians across Japan, we do not have data on plasma levels of ADAMTS13:AC in CTD-patients without TMA. However, such a control study was previously reported by Mannucci et al. (49) in 2003, who showed that SLE-patients without TMA had slightly but significantly reduced plasma levels of ADAMTS13:AC in comparison to normal individuals (Mean ± standard deviation [SD]: 89 ± 33% vs. 107 ± 27, p=0.013). In fact, none of these CTD patients without TMA had severe deficiencies in ADAMTS13:AC or detectable ADAMTS13:INH. Although we are presently unable to address the difference in plasma levels of ADAMTS13:AC between Japanese and Europeans, Rieger et al. (50) confirmed the results of Mannucci et al. (49) by analysing 40 SLE-patients without TMA, and finding that five of their 40 (13%) patients had non-neutralising IgG-autoantibodies against ADAMTS13. Thus, it is also possible that non-inhibitory autoantibodies to ADAMTS13 may play an important role in the pathogenesis of CTD-TMA without severe deficiency of ADAMTS13:AC, possibly through enhanced clearance of ADAMTS13 from the circulation or by blocking the enzyme from the cell surface for efficient cleavage of UL-VWFMs.

We have demonstrated the existence of at least two phenotypic TMAs related to CTDs: a minor population caused by deficient ADAMTS13:AC with neutralising autoantibodies, as typically shown in ai-TTP, and a major population without these autoantibodies. Interestingly, the former group has had better therapeutic outcomes than the latter.

What is known about this topic?

- Acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP) is typically defined as 'severe' deficiency of ADAMTS13 activity due to its IgG-autoantibodies, by the previous assays but with a limited sensitivity (3–5% of normal controls).
- However, the pathogenesis of connective tissue disease-associated thrombotic microangipathies (CTD-TMA) has been largely unknown, because only few reports have shown to have 'severe' deficiency of ADAMTS13 activity, in spite of its quite high prevalence (1–6% of patients with CTDs).

What does this paper add?

- By analysing 64 patients with ai-TTP and 127 patients with CTD-TMA, using a highly sensitive ELISA assay for ADAMTS13 activity, frequency of true 'severe' deficiency of ADAMTS13 activity (<0.5% of normal) was identified in 70% in the former and 17% in the latter.
- CTD-TMAs have been categorised into the following three groups; severe, moderate-to-mild, and subnormal-to-normal activity of ADAMTS13. These 3 groups were comparatively analysed on their clinical and therapeutic outcomes.
- Clinical and therapeutic outcome of patients of CTD-TMA with 'severe' deficiency was almost comparable to that of ai-TTP, but the remaining two groups of CTD-TMAs had apparently poor results. Differential mechanistic pathogenesis of these three groups has been extensively discussed.

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Conflict-of interest disclosure

Y. F. is a member of clinical advisory boards for Baxter BioScience.

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ORIGINAL ARTICLE

A phase II, open-label, sequential-cohort, dose-escalation study of romiplostim in Japanese patients with chronic immune thrombocytopenic purpura

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Abstract This phase II, multicenter, open-label, sequential-cohort, dose-escalation study was designed to evaluate the safety and efficacy of romiplostim, a novel peptibody that increases platelet production, in Japanese patients with chronic immune thrombocytopenic purpura (ITP). Sequential cohorts of four patients each received romiplostim (1, 3, or 6 μg/kg) subcutaneously on days 1 and 8 of the dose-escalation phase. Patients who achieved platelet responses (doubling of baseline platelet counts to $\geq 50 \times 10^9$/L) continued romiplostim weekly during the treatment-continuation phase. Romiplostim produced dose-dependent increases in mean and peak platelet counts. Five patients received romiplostim during the treatment-continuation phase, with platelet counts $\geq 50 \times 10^9$/L maintained in approximately half of the weekly assessments. Romiplostim was well tolerated. No severe, serious, or life-threatening adverse events were reported. No binding antibodies to romiplostim or thrombopoietin were detected. Romiplostim is safe and well tolerated in Japanese patients with chronic ITP and is effective in producing platelet count increases, consistent with the results from studies in non-Japanese patients. On the basis of these findings, a starting dose of 3 μg/kg was recommended for phase III evaluation of romiplostim in Japanese patients with chronic ITP.

Keywords Immune thrombocytopenic purpura · Romiplostim · Thrombopoiesis · Platelet production

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1 Introduction

Chronic immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by antibody-mediated platelet destruction and suboptimal platelet production [1–4]. Initial therapy typically consists of corticosteroids or intravenous immunoglobulins (IVIg), followed by low-dose corticosteroids (prednisone, 1–2 mg/kg/day) if thrombocytopenia persists. Either repeated administrations of IVIg or splenectomy can be used for intolerance or insufficient response to low-dose corticosteroids [1, 3]. These treatments as well as others used in refractory patients, such as rituximab and cyclophosphamide, suppress the rate of platelet destruction [5–7]. However in many cases they are either transiently effective, insufficiently effective, or poorly tolerated. In addition to the platelet destruction, the rate of platelet production is

inadequate in a majority of patients with chronic ITP [4, 8–11]. Accordingly, treatments that increase platelet production may offer the potential for improved control and outcomes in chronic ITP [12]. Platelet production is primarily regulated by thrombopoietin (TPO), which binds to the TPO receptor (c-Mpl) to increase the megakaryocytopoiesis and thrombopoiesis.

The potential clinical benefit of using a thrombopoietic growth factor to treat chronic ITP was initially demonstrated using pegylated recombinant human megakaryocyte growth and development factor (MGDF), a truncated form of human TPO [13]. Administration of MGDF for 7 days increased platelet counts in 3 of 4 Japanese ITP patients and bleeding was decreased. The production of antibodies against MGDF in healthy volunteers that cross-reacted with endogenous TPO [14] resulted in the discontinuation of MGDF clinical studies and led to the development of novel TPO mimetics. Romiplostim (AMG 531) is an Fc-peptide fusion protein (peptibody) that increases platelet production via the same mechanism as endogenous TPO [15, 16]. However, romiplostim does not share sequence homology with TPO. This lack of sequence homology reduces the probability that antibodies to romiplostim, if produced, would cross-react with endogenous TPO and cause further thrombocytopenia [14]. Initial clinical trials with romiplostim in the United States and Europe showed that romiplostim increases platelet counts in healthy volunteers and during short-term use by patients with chronic ITP [17–19]. Recent phase III studies conducted in the United States and Europe showed that romiplostim raised and sustained platelet counts in splenectomized and non-splenectomized patients with chronic ITP during treatment for 24 weeks [20]. In these studies, platelet responses were defined as durable or transient and required that rescue medication had not been administered in the preceding 8 weeks. Durable platelet responses, a very rigorous end point that required platelet counts $\geq 50 \times 10^9$/L for at least 6 of the last 8 weeks of treatment without a need for rescue medication, were achieved significantly more often with romiplostim than with placebo in splenectomized patients (38 vs. 0%; $P = 0.0013$) and non-splenectomized patients (61 vs. 5%; $P < 0.001$). The overall platelet response rate (i.e., either transient or durable responses with platelet counts $\geq 50 \times 10^9$/L for 4 weeks or more) was also significantly higher with romiplostim than placebo in splenectomized patients (79 vs. 0%; $P < 0.0001$) and non-splenectomized patients (88 vs. 14%; $P < 0.0001$). Romiplostim treatment also allowed many patients to reduce or discontinue concomitant ITP therapies and was well tolerated in each of the phase II and III trials in Western countries.

The incidence of chronic ITP in adults in Japan is estimated to be 500–2000 cases annually, a rate of incidence similar to that seen in Western countries [1, 21, 22]. The safety, pharmacodynamics, and pharmacokinetics of romiplostim in Japanese adult patients were demonstrated in a phase I study and were consistent with those seen previously in healthy non-Japanese subjects [21]. In the phase I Japanese study, romiplostim increased platelet counts in a dose-related manner, with four of eight patients who received a dose of 1 μg/kg and seven of eight patients who received a dose of 2 μg/kg having platelet increases ≥ 1.5 times above baseline. The present phase II study was conducted to evaluate the safety and tolerability of romiplostim and its effect on platelet counts in Japanese patients with chronic ITP, and to identify an appropriate starting dose for a phase III study of romiplostim for the treatment of chronic ITP in adult Japanese patients.

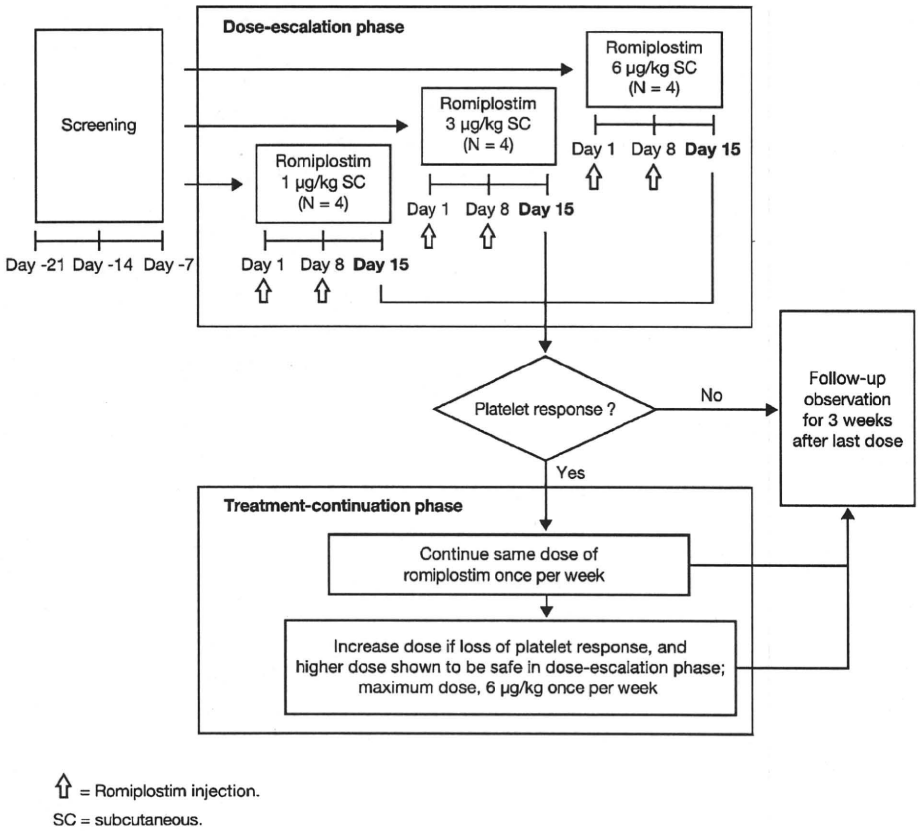
2 Methods

2.1 Study design

This phase II, open-label, sequential-cohort, dose-escalation study was conducted at six centers in Japan and consisted of a 3-week screening period, a 2-week dose-escalation phase and subsequent treatment-continuation phase, and a 3-week follow-up observation period (Fig. 1). It was conducted in accordance with the principles of the Japanese Ministry of Health, Labor and Welfare and International Conference on Harmonization guidelines of Good Clinical Practice. The study protocol and informed consent form were approved by the institutional review board at each study site before any patients were enrolled.

Patients were screened for eligibility over a 3-week period, during which platelet counts were determined each week (days −21, −14, and −7). The baseline platelet count necessary for enrollment was based on the mean of these determinations. Physical examination, vital signs, and laboratory testing were performed at one visit during the screening period. In addition, bone marrow testing was done in all patients aged over 60 years who had not had such testing within the previous 5 years. For the dose-escalation phase, four patients were to be enrolled at each of four sequential dose levels of romiplostim (1, 3, 6, 10 μg/kg). Each dose was administered subcutaneously once weekly for 2 weeks (i.e., days 1 and 8), with no dose adjustments allowed. According to the study protocol, the dose-escalation phase was to be stopped if at least three subjects in a cohort had platelet counts $>450 \times 10^9$/L; at least two subjects in a cohort had platelet counts $>700 \times 10^9$/L; at least one subject in a cohort had platelet counts $>1000 \times 10^9$/L; or if two or more subjects in a cohort had drug-related serious adverse events.

Fig. 1 Study design



Patients who achieved a platelet response, defined as a doubling of the baseline platelet counts to a level $\geq 50 \times 10^9$/L, during the dose-escalation phase were eligible to continue into the treatment-continuation phase. Patients continued to receive romiplostim once weekly at the original dose, with the option of adjusting the dose to achieve platelet counts in a target range of $50–200 \times 10^9$/L. The treatment-continuation phase ended when the final cohort of the dose-escalation phase was completed. At this point, patients entered the follow-up observation phase and had their end of study (EOS) visit 3 weeks after receiving their last dose of romiplostim. Patients entered the observation phase when (1) they failed to respond to romiplostim during the dose-escalation phase, (2) they lost their response during the treatment-continuation phase (defined by two consecutive platelet counts dropping to baseline), or (3) the final dose-escalation cohort was completed. At the end of the study, platelet responders and non-responders were eligible to enter an open-label extension study.

Rescue medications were permitted in the study for a tendency for severe bleeding or if the investigator thought the patient was at immediate risk. These medications were to be given with the intended purpose of raising platelet counts and included IVIg, platelet transfusion, corticosteroids, or an increase in dose or frequency of concurrent corticosteroids. Romiplostim was to be continued in patients who received rescue medications.

2.2 Patients

Japanese patients aged 20–70 years with a diagnosis of ITP for at least 6 months before the first screening visit were eligible if their mean platelet count measured at the three screening visits was $<30 \times 10^9$/L while not receiving any ITP therapy or $<50 \times 10^9$/L while receiving a stable dose of corticosteroids. Eligible patients had received at least one previous treatment for ITP and had Eastern Cooperative Oncology Group (ECOG) performance status 0–2, adequate renal and hepatic function, and a hemoglobin level ≥ 10 g/dL. Patients who were positive for antibodies to *Helicobacter pylori* had to complete one course of *H. pylori* eradication therapy at least 12 weeks before the first screening visit. All patients provided written informed consent.

Patients with a known history of a bone marrow stem cell disorder or abnormal bone marrow findings other than ITP were excluded as were those with arterial thrombosis within the past year, history of venous thrombosis who were receiving anticoagulation therapy, uncontrolled

cardiac disease, uncontrolled hypertension with diastolic blood pressure above 100 mmHg, high risk of thromboembolic events, active malignancy, or major surgery within the past 8 weeks. Patients were excluded if they were currently receiving any treatment for ITP except a stable dose of oral corticosteroids, or if they had received IVIg, high-dose corticosteroid pulse therapy, any drug administered to increase platelet counts, or hematopoietic growth factors within 4 weeks before the first screening visit, or had undergone splenectomy within 12 weeks.

2.3 Assessments

In the safety assessment, all adverse events observed by the investigator or reported by the patient were recorded, and their severity and relationship to study drug were determined by the investigator. Safety was also assessed by clinical laboratory testing (hematology, clinical chemistry, and coagulation), by the measurement of vital signs and electrocardiogram and by physical examination. In addition, blood samples were collected on day 1, at week 7, and then once every 8 weeks during the treatment-continuation phase, and at the EOS visit to test for induction of serum antibodies. A biosensor immunoassay was used initially to detect antibodies against romiplostim, the biologically active peptide portion of romiplostim or TPO. If a sample tested positive, a cell-based bioassay was to be used to test for the presence of neutralizing antibodies against romiplostim or TPO activity on cell growth.

Efficacy was assessed by measuring platelet counts. Parameters measured included platelet response (as defined above), peak platelet count, time to peak platelet count, absolute change from baseline to peak platelet count, and ratio of the peak platelet count to the baseline platelet count (i.e., fold change from baseline to peak) in each cohort of the dose-escalation phase.

2.4 Statistics

The planned sample size was four patients per dose-level cohort. The safety and efficacy analyses included all patients who received at least one dose of romiplostim, and they were conducted separately for the dose-escalation and treatment-continuation phases. The primary end point was the incidence of adverse events, including the presence of antibodies against romiplostim. Adverse events were categorized according to the Medical Dictionary for Regulatory Activities (MedDRA), summarized by severity and relationship to study drug, and evaluated using descriptive statistics. Secondary end points included the proportion of patients achieving a platelet response; proportion of patients with various peak platelet counts (including doubling of baseline counts, absolute counts $\geq 50 \times 10^9$/L, $\geq 100 \times 10^9$/L, and $\geq 450 \times 10^9$/L, and increases $\geq 20 \times 10^9$/L over baseline); and the peak platelet count, time to peak, and absolute and fold change from baseline to peak platelet count in the dose-escalation phase. For the efficacy evaluation, the baseline platelet count was defined as the average of four scheduled determinations (days −21, −14, and −7 during the screening period and day 1 pre-dose). Each efficacy end point was evaluated using descriptive statistics.

3 Results

3.1 Patient disposition and demographics

Four patients were enrolled at each of the first three dose levels (1, 3, 6 μg/kg). One patient in the 6-μg/kg cohort had an excessively high-platelet count (980×10^9/L), and consequently dose escalation to 10 μg/kg in a new cohort of patients was not performed. All 12 patients who were enrolled completed the dose-escalation phase, and five patients entered and completed the treatment-continuation phase (Fig. 2).

The study cohort of 12 patients, all of whom were Japanese per the study protocol, had a mean age of 55.6 years, and included eight females (66.7%) (Table 1). Overall, the mean duration since ITP diagnosis was 10.3 years, and the mean baseline platelet count was 11.8×10^9/L. All patients had ECOG performance status 0, except for one patient in the 3-μg/kg cohort with a performance status of 1. Eleven patients (91.7%) had a history of purpura/petechiae, and nine patients (75.0%) had a history of epistaxis and oral bleeding. Seven patients (58.3%) received stable corticosteroid therapy concomitantly with study drug, and three patients had previously undergone splenectomy. Prior medications for ITP included corticosteroids (91.7%), IVIg (58%), and danazol (42%).

3.2 Dose-escalation phase

The mean platelet count increased with romiplostim dose when measured on days 8, 11, and 15 (Fig. 3). The proportion of patients with platelet responses by day 8, the first time platelet counts were assessed following treatment, increased with romiplostim dose from 0% at 1 μg/kg to 50% at 3 μg/kg and 100% at 6 μg/kg (Table 2). By day 11, one of four patients (25%) treated with romiplostim 1 μg/kg also had a platelet response. Overall, seven of the 12 patients (58.3%) achieved platelet responses, including six of eight patients (75.0%) treated with doses of 3 or 6 μg/kg. As shown in Table 2, romiplostim produced dose-related increases in the other efficacy measures. The mean peak platelet count ranged from 44×10^9/L at 1 μg/kg to 374×10^9/L at 6 μg/kg,

Fig. 2 Patient disposition

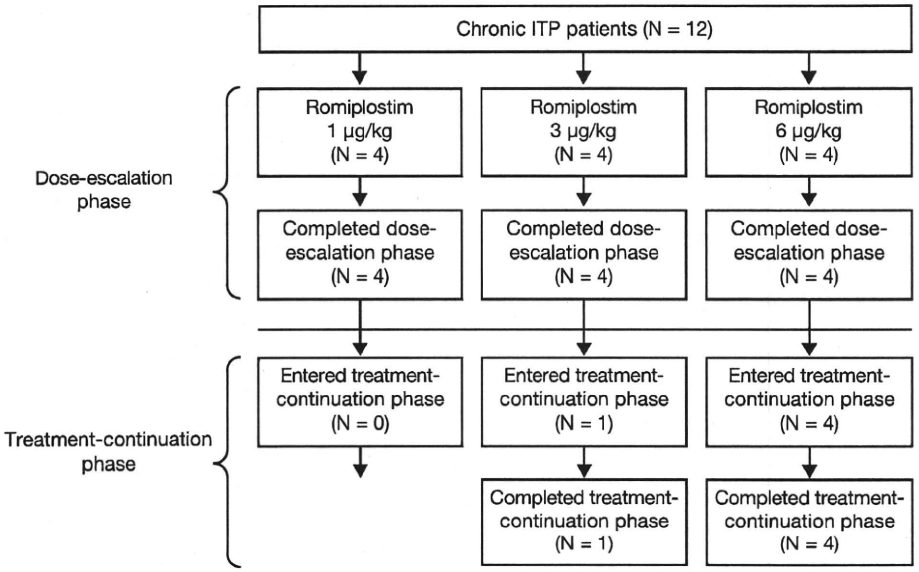


Table 1 Patient demographics and baseline characteristics

Characteristic	Dose cohort			
	1 μg/kg ($n = 4$)	3 μg/kg ($n = 4$)	6 μg/kg ($n = 4$)	Total ($N = 12$)
Sex, *n* (%)				
Female	2 (50.0)	3 (75.0)	3 (75.0)	8 (66.7)
Age (years), mean (SD)	61.5 (1.3)	52.0 (13.7)	53.3 (7.2)	55.6 (9.2)
Years since diagnosis, mean (SD)	11.1 (7.1)	9.0 (4.7)	10.9 (5.9)	10.3 (5.5)
Baseline platelet counts $\times 10^9$/L				
Mean (SD)	9.8 (5.8)	8.8 (9.4)	16.8 (13.0)	11.8 (9.7)
Range	4–15	3–23	5–31	3–31
Corticosteroids, *n* (%)				
Prior use	3 (75.0)	4 (100)	4 (100)	11 (91.7)
Concurrent use	2 (50.0)	3 (75.0)	2 (50.0)	7 (58.3)
Previous splenectomy, *n* (%)	1 (25.0)	0 (0)	2 (50.0)	3 (25.0)
Number of prior ITP therapies[a], mean (SD)	4.5 (3.3)	4.0 (2.1)	3.5 (2.3)	4.0 (2.6)

[a] Excluding splenectomy

with the proportion of patients achieving various cut points for response, including platelet count $\geq 20 \times 10^9$/L above baseline and platelet count $\geq 50 \times 10^9$/L or $\times 100 \times 10^9$/L, also increasing with romiplostim dose. All four patients in the 6-μg/kg cohort achieved these levels of peak response. When compared with the platelet count at baseline, the absolute change ranged from 34×10^9/L at 1 μg/kg to 357×10^9/L at 6 μg/kg, with the peak values representing a mean 4.1- to 26.2-fold increase above baseline. The mean time to peak response was approximately 13 days and did not differ by dose.

All 12 patients received both scheduled doses of romiplostim. The mean (SD) total dose of romiplostim was 116 ± 18 μg in the 1-μg/kg cohort, 360 ± 70 μg in the 3-μg/kg cohort, and 718 ± 116 μg/kg in the 6-μg/kg cohort. None of the patients received rescue medication.

Romiplostim was well tolerated. Overall, eight patients (66.7%) experienced at least one adverse event, most of which were mild in severity (Table 3). No severe, serious, or life-threatening adverse events were reported, and no patient withdrew due to an adverse event. Six patients (50.0%) had treatment-related adverse events, most commonly headache ($n = 3$; 25.0%). There was no apparent relationship between the romiplostim dose and the incidence of treatment-related adverse events. Other than changes in platelet counts, there were no clinically significant changes in other serum chemistry, hematology, or coagulation laboratory values during the course of the

dose-escalation phase. Similarly, no clinically significant changes in vital signs were observed.

Four patients had adverse events of bleeding. One patient in the 1-μg/kg cohort experienced epistaxis on day 21 (i.e., 13 days after the last dose of romiplostim). The platelet count was not recorded on the day of epistaxis, but was 9 × 10^9/L on the following day when the patient also experienced mouth hemorrhage. Another patient in the 1-μg/kg group had purpura and tongue hematoma on day 8, which was the day of the second romiplostim dose. This patient never achieved a response to romiplostim and had a platelet count of 2 × 10^9/L at the time of the bleeding event. One patient in the 3-μg/kg cohort experienced epistaxis on day 12. This subject had platelet counts of 199 × 10^9/L and 361 × 10^9/L when measured 2 days before and 3 days after the bleeding event, respectively. Finally, one patient in the 6-μg/kg cohort experienced purpura on day 31 (23 days after the second romiplostim dose); the platelet count was 17 × 10^9/L on the day of the bleeding event and 38 × 10^9/L when measured 5 days later.

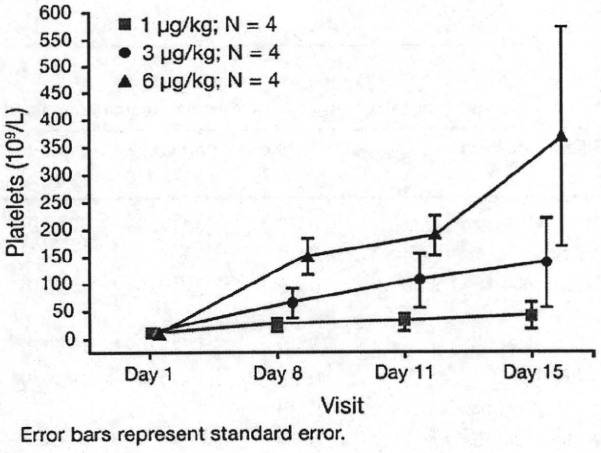


Fig. 3 Mean platelet counts in the dose-escalation phase by romiplostim dose

All patients had a baseline sample and at least one post-baseline sample for testing for the presence of antibodies induced by romiplostim exposure. All tested samples were negative for binding antibodies to romiplostim, the active peptide portion of romiplostim, and TPO in the immunoassay, and therefore no samples were evaluated in the cell-based bioassay.

3.3 Treatment-continuation phase

Five patients entered the treatment-continuation phase, including one patient from the 3-μg/kg cohort and all four patients from the 6-μg/kg cohort. The patient who received romiplostim 3 μg/kg had a baseline platelet count of 6 × 10^9/L and values ranging from 19 to 115 × 10^9/L during the treatment-continuation phase. Approximately, half of these weekly assessments showed platelet counts of >50 × 10^9/L (Fig. 4). Mean platelet counts in the four patients who received romiplostim 6 μg/kg generally remained ≥100 × 10^9/L through week 13, although values varied widely among the individual patients. By week 14, mean platelet counts had declined to 24 × 10^9/L, and no additional doses of romiplostim were given under the study

Table 2 Platelet responses to romiplostim in the dose-escalation phase

Efficacy measure	Dose cohort			
	1 μg/kg (n = 4)	3 μg/kg (n = 4)	6 μg/kg (n = 4)	Total (N = 12)
Platelet response, n (%)	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Day 8	0 (0)	2 (50.0)	4 (100)	6 (50.0)
Day 11	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Day 15	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Platelet count ≥20 × 10^9/L over baseline, n (%)	2 (50.0)	3 (75.0)	4 (100)	9 (75.0)
Platelet count ≥50 × 10^9/L, n (%)	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Platelet count ≥100 × 10^9/L, n (%)	1 (25.0)	2 (50.0)	4 (100)	7 (58.3)
Platelet count ≥450 × 10^9/L, n (%)	0 (0)	0 (0)	1 (25.0)	1 (8.3)
Peak platelet count × 10^9/L				
Mean (SE)	44.0 (24.6)	145.8 (80.8)	374.3 (202.1)	188.0 (78.1)
Range	5–116	8–361	153–980	5–980
Change from baseline in peak platelet count				
Fold increase, mean (SE)	4.1 (1.6)	15.9 (6.3)	26.2 (7.2)	15.4 (4.0)
Absolute change × 10^9/L, mean (SE)	34.3 (23.0)	137.0 (76.5)	357.4 (199.4)	176.2 (76.5)
Time to peak platelet count—days, mean (SE)	13.3 (1.8)	11.5 (1.4)	14.0 (1.0)	12.9 (0.8)

Table 3 Summary of safety during the dose-escalation phase

Safety parameter, *n* (%)	Dose cohort			
	1 μg/kg ($n = 4$)	3 μg/kg ($n = 4$)	6 μg/kg ($n = 4$)	Total ($N = 12$)
At least 1 adverse event	3 (75.0)	3 (75.0)	2 (50.0)	8 (66.7)
Serious or severe adverse event	0 (0)	0 (0)	0 (0)	0 (0)
Treatment-related adverse event	1 (25.0)	3 (75.0)	2 (50.0)	6 (50.0)
Headache	1 (25.0)	1 (25.0)	1 (25.0)	3 (25.0)
Fatigue	1 (25.0)	0 (0)	0 (0)	1 (8.3)
Back pain	0 (0)	1 (25.0)	0 (0)	1 (8.3)
Muscle tightness	0 (0)	1 (25.0)	0 (0)	1 (8.3)
Flushing	0 (0)	0 (0)	1 (25.0)	1 (8.3)
Withdrawals due to adverse events	0 (0)	0 (0)	0 (0)	0 (0)
Rescue medication use	0 (0)	0 (0)	0 (0)	0 (0)
Anti-romiplostim neutralizing antibodies	0 (0)	0 (0)	0 (0)	0 (0)

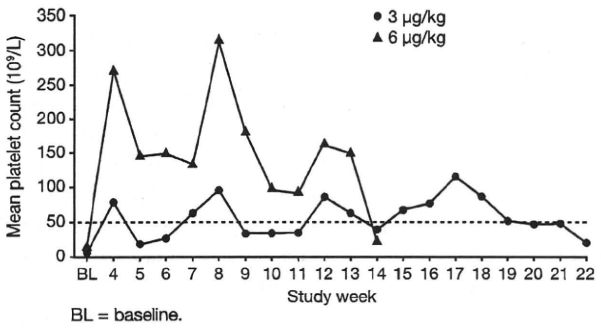


Fig. 4 Mean platelet counts in the treatment-continuation phase by romiplostim dose

protocol due to the completion of the study. However, all five patients in the treatment-continuation phase subsequently entered an open-label extension study to continue romiplostim treatment.

The patients who participated in the treatment-continuation phase received a mean of 10 ± 4.8 doses of romiplostim. The patient in the 3-μg/kg cohort received 18 doses of romiplostim (all doses at 3 μg/kg; total dose 3240 μg). The four patients in the 6-μg/kg cohort received a mean of 8 ± 2 doses (one patient had all doses at 6 μg/kg; three patients had doses adjusted ranging from 0 to 5 μg/kg; mean total dose 2581 μg; range 1775–3030 μg). None of the subjects received rescue medication during the treatment-continuation phase.

The safety and tolerability of romiplostim was comparable to that observed during the dose-escalation phase. No severe, serious, or life-threatening adverse events were reported, and no patient withdrew due to an adverse event. Three patients (60%) had adverse events, most frequently contusion in two subjects receiving the 6-μg/kg dose. Two of the five patients had treatment-related adverse events, with malaise, arthralgia, and contact dermatitis each occurring in one patient treated with 6 μg/kg (Table 4). There were no adverse events of bleeding. Other than the changes in platelet counts, no clinically significant changes were noted in other serum chemistry, hematology, or coagulation laboratory values or clinically significant changes in vital signs during the treatment-escalation phase. Similarly, binding antibodies were not detected by immunoassay in any of the patients.

Table 4 Summary of safety during the treatment-continuation phase

Safety parameter, *n* (%)	3 μg/kg ($n = 1$)	6 μg/kg ($n = 4$)	Total ($N = 5$)
At least 1 adverse event	0 (0)	3 (75.0)	3 (60.0)
Serious or severe adverse event	0 (0)	0 (0)	0 (0)
Treatment-related adverse event	0 (0)	2 (50.0)	2 (40.0)
Malaise	0 (0)	1 (25.0)	1 (20.0)
Arthralgia	0 (0)	1 (25.0)	1 (20.0)
Contact dermatitis	0 (0)	1 (25.0)	1 (20.0)
Withdrawals due to adverse events	0 (0)	0 (0)	0 (0)
Rescue medication use	0 (0)	0 (0)	0 (0)
Anti-romiplostim neutralizing antibodies	0 (0)	0 (0)	0 (0)

4 Discussion

The results of this phase II study show that romiplostim treatment appears to be safe and well tolerated by Japanese patients with chronic ITP. Moreover, this study provides evidence that romiplostim is effective in stimulating platelet count increases in these Japanese patients. All four patients treated with a dose of 6 μg/kg and one half of