who fail to meet pre-defined clinical end-points, and that beneficial effects were observed in patients treated with epoprostenol by the sequential addition of ERA or PDE5I [26].

Epoprostenol therapy was well tolerated in general by all of our patients with PAH–CTD. Flushing, headache, and diarrhea were common and tended to be dose-dependent, but these could be managed by adjusting the dose-escalation schedule. However, particular attention should be paid to catheter-related infection. None of the patients died of this complication during the observation period of this study, but we recently experienced a patient with SLE who died of sepsis after repeated episodes of catheter-related infection. We failed to identify risk factors for this serious complication, possibly because of insufficient statistical power due to the small number of patients enrolled in this study. It is possible that an immunodeficient status and thinning of the skin due to the use of corticosteroids contribute to this complication.

Patients with advanced ILD were excluded from our study, but we generally do not use epoprostenol for patients with PH and advanced ILD because ventilation/perfusion mismatch is increased by the use of intravenous epoprostenol, thereby worsening oxygenation [27]. This unfavorable effect has, in fact, been reported in SSc patients with PH and concomitant ILD [28].

There are several limitations to our study. First, the number of patients was small, and all the patients were treated at a single PH referral center. Therefore, we acknowledge that our data on the outcomes of patients treated with epoprostenol therapy might not be representative of all patients with PAH–CTD. Second, because some of the patients received both epoprostenol and another therapy, such as PAH drugs and immunosuppressive therapy, it was not possible to attribute long-term outcomes to a specific therapeutic agent. Finally, this study included 13 patients who were diagnosed as having PAH and treated before 2005. At that time, beraprost and epoprostenol were the only PAH drugs available in Japan. Thus, the observed long-term survival rates may not reflect the current treatment environment in which a series of oral ERAs and PDE5Is are available. This also means that, in our cohort, the efficacy of epoprostenol could be assessed with minimal confounding effects from ERA and/or PDE5I.

In summary, based on our results, we conclude that epoprostenol treatment is effective for patients with advanced PAH irrespective of the underlying CTDs, but its efficacy in patients with end-stage PAH is limited. The maximum therapeutic benefit of epoprostenol is obtained when it is initiated before patients develop end-stage PAH, and its dosage is increased as rapidly as possible during the first 6 months of treatment. This strategy needs to be validated in a larger cohort.

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Conflict of interest None.

References

1. Mathai SC, Hassoun PM. Pulmonary arterial hypertension in connective tissue diseases. Heart Fail Clin. 2012;8:413–25.
2. Chung L, Liu J, Parsons L, Hassoun PM, McGoon M, Badesch DB, et al. Characterization of connective tissue disease-associated pulmonary arterial hypertension from REVEAL: identifying systemic sclerosis as a unique phenotype. Chest. 2010;138:1383–94.
3. Shirai Y, Yasuoka H, Okano Y, Takeuchi T, Satoh T, Kuwana M. Clinical characteristics and survival of Japanese patients with connective tissue disease and pulmonary arterial hypertension: a single-center cohort. Rheumatology (Oxford).2012;51:1846–54
4. Mathai SC, Hummers LK, Champion HC, Wigley FM, Zaiman A, Hassoun PM, et al. Survival in pulmonary hypertension associated with the scleroderma spectrum of diseases: impact of interstitial lung disease. Arthritis Rheum. 2009;60:569–77.
5. Hachulla E, Carpentier P, Gressin V, Diot E, Allanore Y, Sibilia J, et al. Risk factors for death and the 3-year survival of patients with systemic sclerosis: the French ItinérAIR-Sclérodermie study. Rheumatology. 2009;48:304–8.
6. Condliffe R. Corris PA, Gibbs JS, Vrapi F, et al. Connective tissue disease-associated pulmonary arterial hypertension in the modern treatment era. Am J Respir Crit Care Med. 2009;179:151–7.
7. Hesselstrand R, Wildt M, Ekmehag B, Wuttge DM, Scheja A. Survival in patients with pulmonary arterial hypertension associated with systemic sclerosis from a Swedish single centre: prognosis still poor and prediction difficult. Scand J Rheumatol. 2011;40:127–32.
8. Zhang R, Dai LZ, Xie WP, Yu ZX, Wu BX, Pan L, et al. Survival of Chinese patients with pulmonary arterial hypertension in the modern management era. Chest. 2011;140:301–9.
9. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary hypertension. The Primary Pulmonary Hypertension Study Group. N Engl J Med. 1996;334:296–302.
10. Badesch DB, Tapson VF, McGoon MD, Brundage BH, Rubin LJ, Wigley FM, et al. Continuous intravenous epoprostenol for pulmonary hypertension due to the scleroderma spectrum of disease. A randomized, controlled trial. Ann Intern Med. 2000;132:425–34.
11. Humbert M, Sanchez O, Fartoukh M, Jagot JL, Le Gall C, Sitbon O, et al. Short-term and long-term epoprostenol (prostacyclin) therapy in pulmonary hypertension secondary to connective tissue diseases: results of a pilot study. Eur Respir J. 1999;13:1351–6.
12. Robbins IM, Gaine SP, Schilz R, Tapson VF, Rubin LJ, Loyd JE. Epoprostenol for treatment of pulmonary hypertension in patients with systemic lupus erythematosus. Chest. 2000;117:14–8.
13. McLaughlin VV, Shillington A, Rich S. Survival in primary pulmonary hypertension: the impact of epoprostenol therapy. Circulation. 2002;106:1477–82.
14. Sitbon O, Humbert M, Nunes H, Parent F, Garcia G, Hervé P, et al. Long-term intravenous epoprostenol infusion in primary

pulmonary hypertension: prognostic factors and survival. J Am Coll Cardiol. 2002;40:780–8.
15. Barst RJ, Gibbs JS, Ghofrani HA, Hoeper MM, McLaughlin VV, Rubin LJ, et al. Updated evidence-based treatment algorithm in pulmonary arterial hypertension. J Am Coll Cardiol. 2009;54: S78–84.
16. Kuhn KP, Byrne DW, Arboqast PG, Doyle TP, Loyd JE, Robbins IM. Outcome in 91 consecutive patients with pulmonary arterial hypertension receiving epoprostenol. Am J Resipr Crit Care Med. 2003;167:580–6.
17. Badesch DB, McGoon MD, Barst RJ, et al. Longterm survival among patients with scleroderma-associated pulmonary arterial hypertension treated with intravenous epoprostenol. J Rheumatol. 2009;36:2244–9.
18. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40:1725.
19. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum. 1980;23:581–590.
20. Kasukawa R, Tojo T, Miyawaki S. Preliminary diagnostic criteria for classification of MCTD. In: Kasukawa R, Sharp GC, editors. Mixed connective tissue disease and anti-nuclear antibodies. Elsevier: Amsterdam; 1987. p. 23–32.
21. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 2002;61:554–8.
22. Badesch DB, Champion HC, Sanchez MA, Hoeper MM, Loyd JE, Manes A, et al. Diagnosis and assessment of pulmonary arterial hypertension. J Am Coll Cardiol. 2009;30:S55–66.
23. Kuwana M, Kaburaki J, Okano Y, Tojo T, Homma M. Clinical and prognostic associations based on serum antinuclear antibodies in Japanese patients with systemic sclerosis. Arthritis Rheum. 1994;37:75–83.
24. Klings E, Hill N, Ieong M, Simms R, Korn J, Farber H. Systemic sclerosis-associated pulmonary hypertension. Arthritis Rheum. 1999;42:2638–45.
25. Akagi S, Nakamura K, Miyaji K, Ogawa A, Kusano KF, Ito H, et al. Marked hemodynamic improvements by high-dose epoprostenol therapy in patients with idiopathic pulmonary arterial hypertension. Circ J. 2010;74:2200–5.
26. Johnson SR, Brode SK, Mielniczuk LM, Granton JT. Dual therapy in IPAH and SSc-PAH. A qualitative systematic review. Respir Med. 2012;106:730–9.
27. Ghofrani HA, Wiedemann R, Rose F, Schemuly RT, Olschewski H, Weissmann N, et al. Sildenafil for treatment of lung fibrosis and pulmonary hypertension: a randomised controlled trial. Lancet. 2002;360:895–900.
28. Strange C, Bolster M, Mazur J, Taylor M, Gossage JR, Silver R. Hemodynamic effects of epoprostenol in patients with systemic sclerosis and pulmonary hypertension. Chest. 2000;118:1077–82.

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Analysis of genetic and predisposing factors in Japanese patients with atypical hemolytic uremic syndrome

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ABSTRACT

Hemolytic uremic syndrome (HUS) is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment. Approximately 10% of cases are classified as atypical due to the absence of Shiga toxin-producing bacteria as a trigger. Uncontrolled activation of the complement system plays a role in the pathogenesis of atypical HUS (aHUS). Although many genetic studies on aHUS have been published in recent years, only limited data has been gathered in Asian countries. We analyzed the genetic variants of 6 candidate genes and the gene deletion in complement factor H (CFH) and CFH-related genes, examined the prevalence of CFH autoantibodies and evaluated the genotype-phenotype relationship in 10 Japanese patients with aHUS. We identified 7 causative or potentially causative mutations in *CFH* (p.R1215Q), *C3* (p.R425C, p.S562L, and p.I1157T), membrane cofactor protein (p.Y189D and p.A359V) and thrombomodulin (p.T500M) in 8 out of 10 patients. All 7 of the mutations were heterozygous and four of them were novel. Two patients carried *CFH* p.R1215Q and 3 other patients carried *C3* p.I1157T. One patient had 2 causative mutations in different genes. One patient was a compound heterozygote of the 2 *MCP* mutations. The patients carrying mutations in *CFH* or *C3* had a high frequency of relapse and a worse prognosis. One patient had CFH autoantibodies. The present study identified the cause of aHUS in 9

Abbreviations: aHUS, atypical hemolytic uremic syndrome; CFH, complement factor H; C3, complement component 3; MCP, membrane cofactor protein; CFI, complement factor I; CFB, complement factor B; CFD, complement factor D; THBD, thrombomodulin; CFHRs, CFH related genes; SCR, short consensus repeat; RCA, regulators of complement activation; RFLP, restriction fragment length polymorphism; MLPA, multiplex ligation-dependent probe amplification; URTI, upper respiratory tract infection.

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out of 10 Japanese patients. Since the phenotype-genotype correlation of aHUS has clinical significance in predicting renal recovery and transplant outcome, a comprehensively accurate assessment of molecular variation would be necessary for the proper management of aHUS patients in Japan.

1. Introduction

Hemolytic uremic syndrome (HUS) is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment (Boyce et al., 1995). Approximately 10% of the cases are classified as atypical due to the absence of Shiga toxin-producing bacteria infection as a trigger (Noris and Remuzzi, 2009). Compared to typical HUS, atypical HUS (aHUS, OMIM #235400) has a much poorer prognosis, with up to half of the patients progressing to end-stage renal disease, and a higher mortality (Tarr et al., 2005).

The alternative pathway of the complement system is a natural defense system against invasive microbial attack, in which complement component C3 (C3), the central complement protein, is hydrolyzed to C3b and directly binds to the microbe for opsonization or for the subsequent activation of the complement pathway (Roumenina et al., 2011). When C3b binds to the host cells, the further activation of the complement system is stringently limited by several endogenous complement regulatory proteins which are present on the surface of the host cells (Sethi & Fervenza, 2012). Complement factor H (CFH) and membrane cofactor protein (MCP or CD46) are the regulators in the complement pathway. Both proteins can accelerate the complement factor I (CFI)-mediated proteolytic inactivation of C3b and C4b. CFH can also inhibit the formation of the C3 convertase, C3bBb, by competing with complement factor B (CFB) for binding to C3b and thereby accelerate the decay of C3bBb simultaneously (Roumenina et al., 2011; Sethi and Fervenza, 2012). Thrombomodulin, an endothelial anticoagulant glycoprotein encoded by *THBD*, also functions as a cofactor for the CFI-mediated C3b inactivation, and mutations of *THBD* predispose to aHUS (Delvaeye et al., 2009).

Maintenance of the complement system involves a balance between activation and regulation. Uncontrolled activation of the alternative pathway of the complement system plays a role in the pathogenesis of aHUS. More than half of the patients with aHUS have mutations of genes involved in the alternative pathway of the complement system (Noris and Remuzzi, 2009). Mutations with loss-of-function of regulators (*CFH, CFI, MCP*, and *THBD*) (Delvaeye et al., 2009; Noris et al., 2010; Richards et al., 2003; Sellier-Leclerc et al., 2007) and gain-of-function of key complement components (*C3* and *CFB*) (Fremeaux-Bacchi et al., 2008; Goicoechea de Jorge et al., 2007) have been found to predispose to aHUS. In addition, genomic deletions in the regulators of complement activation (RCA) located on chromosome 1q32 are reportedly associated with the occurrence of aHUS due to the high homology among *CFH* and 5 CFH-related genes (*CFHR3, CFHR1, CFHR4, CFHR2*, and *CFHR5* lie in tandem at 1q32) (Zipfel et al., 2007). In particular, deletion of *CFHR3* and *CFHR1* as a result of non-allelic homologous recombination has been linked to a risk of aHUS (Venables et al., 2006), sometimes together with the presence of CFH autoantibodies (Jozsi et al., 2008; Skerka et al., 2009).

A normal plasma level of complement proteins does not preclude the presence of a mutation in these genes. More importantly, genotype–phenotype correlations of aHUS have clinical significance in predicting renal recovery and transplant outcome (Noris et al., 2010). Therefore, it is important to perform genetic screening of these genes in patients with aHUS. In this study, we described the clinical phenotypes in 10 Japanese aHUS patients, sequenced the 6 candidate genes *CFH, MCP, CFI, C3, CFB*, and *THBD*, examined the gene deletion of *CFH* and *CFHRs* in the RCA region, evaluated the penetrance of genetic abnormalities, and finally determined the genotype–phenotype correlations.

2. Materials and methods

2.1. Patients

Ten Japanese patients with aHUS were investigated in this study; 8 of them were sporadic and the other two were from one family. Diagnosis of aHUS was defined by the simultaneous occurrence of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure without association to Shiga toxin (Ariceta et al., 2009). Clinical events preceding the acute HUS episode were recorded. Laboratory data were collected. The study was approved by the Institutional Review Board of each institution. Written informed consent was obtained from all of the participants.

2.2. Complement analyses

Serum C3 and C4 levels were measured by nephelometry. The CFH antigen level was determined by a rocket-immunoelectrophoresis method using pooled plasma of healthy individuals as 100%. The normal ranges of C3, C4, and CFH were 86–160 mg/dl, 14–49 mg/dl, and 50–150%, respectively.

2.3. ADAMTS13 activity assay

ADAMTS13 activity was measured by a chromogenic ADAMTS13-act-ELISA using a glutathione-conjugated VWF73 peptide as the substrate (Kato et al., 2006).

2.4. Hemolytic assay

Resuspended sheep red blood cells (Japan Lamb, Japan) were incubated with a dilution series of a patient plasma sample at 37 °C for 30 min, and the level of hemoglobin release from the red blood cells was measured by the absorbance at 414 nm (A_{414}) (Sanchez-Corral et al., 2004). The absorbance obtained from the addition of an excess amount of a neutralizing antibody against CFH was defined as 100%. The characterization of the neutralizing antibody against CFH will be described elsewhere. The hemolysis activity of the patients was expressed as the percentage obtained using A_{414} taken from the patient to that obtained using the neutralizing antibody against CFH. A value of more than 50% was regarded as apparent hemolysis.

2.5. Autoantibody against CFH

The autoantibody was examined by the Western blot method(Moore et al., 2010). Purified CFH was electrophoresed on a 5% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. After blocking with 5% dried milk, the membrane was cut into 0.5-cm wide strips and each strip was incubated with the 100-fold diluted patient plasma sample overnight at 4 °C. Horseradish peroxidase-labeled goat anti-human IgG antibody was used as the secondary antibody and bound autoantibodies were visualized by an enhanced chemiluminescence substrate (Western Lightning-ECL, PerkinElmer, Japan).

2.6. Mutation screening

Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen, Germany) from peripheral blood leukocytes of patients and their family members. The coding exons and the intronic flanking regions of *CFH* (NM_000186.3), *C3* (NM_000064.2), *MCP* (NM_002389.4), *CFI* (NM_000204.3), *CFB* (NM_001710.5) and *THBD* (NM_000361.2) were amplified by the polymerase chain reaction. The sequences of gene-specific primers and the polymerase chain reaction conditions are listed in Supplementary Table 1. A routine sequencing reaction was carried out in both directions. The A of the ATG translation initiation start site was designated as position +1 and the initial Met was denoted as +1. The potential pathogenicity of missense mutations was examined by several programs for predicting the functional significance of missense mutations; these were PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), AGVGD (http://agvgd.iarc.fr/cgi-bin/agvgd_output.cgi), SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) and PMut (http://mmb.pcb.ub.es/PMut/).

2.7. Restriction fragment length polymorphism (RFLP) analysis

The RFLP analysis was applied for confirmation of mutations in the family members. The amplified DNA fragments were digested with a restriction enzyme (New England Biolabs, USA) (Table 1). The digests were electrophoresed to determine the genotypes according to the cleaved bands.

2.8. Screening for gene deletions

Multiplex ligation-dependent probe amplification (MLPA) analysis was used to screen the gene deletions in the RCA region on chromosome 1q32 using a commercially available kit (MLPA kit P236-A2; MRC-Holland, the Netherlands). The relative dosage ratio was calculated by Coffalyser v9.4. The probe ratios of deletions should be below 0.7.

3. Results

The clinical features and laboratory data of the 10 patients with aHUS are summarized in Table 2. The parents of all patients were non-consanguineous. Plasma ADAMTS13 activity was within the range of 29–119% in all patients. All the patients showed no signs for infection of Shiga toxin-producing *Escherichia coli*. The first episode of aHUS occurred at childhood (≤10 yr) in 7 patients. Nine cases had probable triggering events. The plasma C3 level was low in patients X1, GG1, HH1 and JJ1. The plasma C4 and CFH levels were in the normal range except in the case of patient HH1, who exhibited a mild decrease in C4. Patients X1, GG1, and II1 showed apparent hemolytic activity against the sheep erythrocytes. The presence of CFH autoantibody was confirmed in only one patient (GG1) (Fig. 1). Five patients had experienced relapses by the most recent follow-up. Five patients progressed to end-stage renal disease and could not be maintained without hemodialysis or peritoneal dialysis.

DNA sequencing of 6 candidate genes identified 17 missense mutations in 10 aHUS patients (Table 2). We considered that 3 of the missense mutations were causative for aHUS, 4 of the novel missense mutations were potentially causative, as described in the results of each proband, and the remaining 10 missense mutations were likely neutral. The detailed characteristics of causative or potentially causative mutations are summarized in Table 3. All of the causative or potentially causative mutations were heterozygous. The causative mutations in the family members were confirmed by the RFLP analysis and were inherited from their unaffected father or mother (Fig. 2). Gene deletions of *CFH* and *CFHRs* in the RCA region were not found in any of the aHUS probands by the MLPA analysis (Table 2).

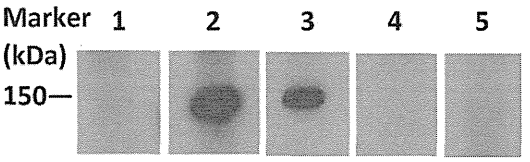

Fig. 1. Detection of CFH autoantibody in family GG. Purified CFH was electrophoresed on a 5% SDS–polyacrylamide gel and transferred to the polyvinylidene fluoride membrane. The membrane was cut into 0.5-cm wide strips and incubated with the diluted plasma sample. Horseradish peroxidase-labeled goat anti-human IgG antibody was used to detect the bound autoantibody. Lane 1, CFH autoantibody-negative plasma; lane 2, CFH autoantibody-positive plasma; lane 3, plasma from patient GG1; lane 4, plasma from patient GG1's father; lane 5, plasma from patient GG1's mother.

3.1. Patient X1

In this male patient, the initial presentation of aHUS was observed after episodes of vomiting, diarrhea and hematuria at 22 years of age (Table 2). At that time, he progressed to anuria. He was treated with hemodialysis three times per week together with drug therapy. At 30 years of age he received a live relative kidney transplantation, but at only 3 weeks after transplantation a renal biopsy of the allograft showed evidence of thrombotic microangiopathy, indicating aHUS had recurred. He received plasma exchanges five times in a week and then gradually tapered to once every two weeks. He is now undergoing treatment with eculizumab, a recombinant humanized monoclonal antibody that specifically binds to complement protein C5, preventing the generation of the cytotoxic membrane-attack complex, C5b-9. Currently, his creatinine level is mildly elevated (2.0–2.5 mg/dl, equal to 177–221 μmol/L).

He had a causative mutation, p.R1215Q, in the short consensus repeats (SCR) 20 domain of *CFH*. He inherited this mutation from his unaffected father (Tables 2 and 3, Fig. 2). Both the patient and his father showed apparently enhanced hemolytic activity.

3.2. Patient AA1

This male patient showed his first overt clinical signs of thrombotic microangiopathy with some petechiae on the face and body at 3 years of age after a cold (Table 2). Then he experienced 6 recurrences of aHUS at the ages of 9, 15, 18, 22, and 29 (twice), with each of these episodes being triggered by upper respiratory tract infection (URTI) or influenza A virus. At the first bout, when he was 29 years old, his laboratory data were improved after 4 plasma exchanges. At the second bout triggered by influenza A, his renal function was worse than that in the first instance, so he was treated with 12 plasma exchanges and 5 rounds of hemodialysis. In each case, his renal function was recovered by prompt treatment after onset.

He had a causative mutation p.I1157T in the thioester-containing domain of *C3*. His unaffected father was a heterozygote for this mutation (Tables 2 and 3, Fig. 2). His hemolytic activity was not enhanced.

3.3. Patient CC1

This male patient developed aHUS at 4 years of age after URTI with palpebral edema and ecchymosis on both his legs and buttocks. He obtained a complete remission only by routine and supportive treatment (Table 2). No causative mutations were identified in the 6 genes sequenced. His hemolytic activity was not enhanced.

Table 1
Restriction fragment length polymorphism (RFLP) assay for causative or potentially causative mutations.

Gene	Reference sequence	Exon	Amino acid change	Restriction enzyme[a]	Allele cut	Forward primer (5′-3′)	Reverse primer (5′-3′)
CFH	NM_000186.3	23	R1215Q	HpyCH4 V	1215Q	atccgtgtgtaatatcccgaga	gcacaagttggatactccagt
C3	NM_000064.2	12	R425C	Hha I	Wild-type	caattcccaggtctcaggga	gagagaaaaggagaaaggg
		13	S562L	Ban II	Wild-type	caattcccaggtctcaggga	gagagaaaaggagaaaggg
		27	I1157T	Ssp I	Wild-type	gcctttgttctcatctcgctgc-aggaggctaaaaata[b]	ctggggataataagagtgactt-acctttcaggctgc
MCP	NM_002389.4	5	Y189D	Sfc I	Wild-type	gtgaagtagaagtatttgagta-tcttgatgcagtaacc[b]	gatgaaactatttacaaaatgttt-ccatagtatttacaaatg
		12	A359V	HpyCH4 V	Wild-type	ggggagttggatttagatagca	ggtaggacaaactaatgcaggc
THBD	NM_000361	1	T500M	BsaH I	Wild-type	cactgctaccctaactacgacct	taaggtgctttggtagcaaagctg

[a] All of the restriction enzymes were available from New England Biolabs (MA, USA) and we used the reaction conditions recommended by the instructions.
[b] The underlined bases in the primer were mismatched with the wild-type sequence in order to introduce the restriction enzyme site.

3.4. Patient DD1

This male patient developed aHUS at 6 years of age, triggered by infection with influenza A virus (Table 2). He had clear thrombocytopenia (platelet count, 20×10^9/L) and hemolytic anemia (hemoglobin, 10 g/dL; lactate dehydrogenase, 3884 U/L) with schistocytes. His creatinine level was 0.9 mg/dL, equal to 79.6 mmol/L on admission, and it increased to 2.85 mg/dL, equal to 251.9 mmol/L. It is noteworthy that neurological abnormalities were also detected. His serological indexes were recovered after treatment with consecutive plasma exchange for 3 days and continuous hemodiafiltration for 7 days.

He had a causative mutation p.Y189D in the SCR3 domain of *MCP* and a potentially causative mutation p.A359V in the transmembrane region of *MCP*. His father and his younger brother had the p.Y189D mutation and his mother had the p.A359V mutation (Tables 2 and 3, Fig. 2). Therefore, the proband was a compound heterozygote for the p.Y189D and p.A359V mutations in *MCP*. None of the family members except for the proband showed any signs of aHUS. His hemolytic activity was not enhanced.

3.5. Patient FF1

This female patient was diagnosed with aHUS at 2 years of age after initial symptoms of palpebral edema and ecchymosis on both her legs appeared. Anemia and thrombocytopenia were improved by transfusion of erythrocyte concentrate and platelets. Her renal function could not be maintained without hemodialysis at that time. She has been treated with peritoneal dialysis for 2 years since her discharge.

She had a potentially causative mutation p.S562L in the β chain of *C3*. The unaffected mother and younger brother carried this mutation (Tables 2 and 3, Fig. 2). Her hemolytic activity was not enhanced.

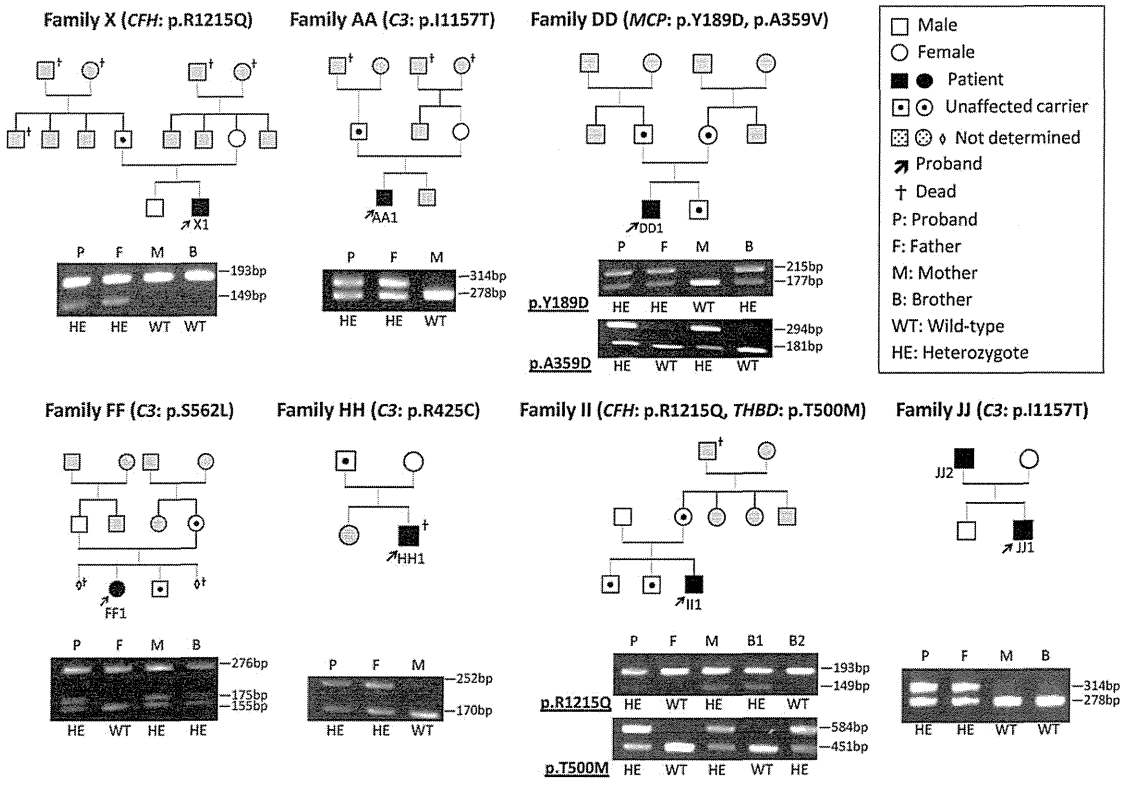

Fig. 2. Family pedigree of 8 patients with aHUS carrying causative or potentially causative mutations. Restriction fragment length polymorphism (RFLP) analyses of causative or potentially causative mutations are shown. The wild-type (WT) and heterozygote (HE) are distinguished by the electrophoretogram after digestion with the corresponding restriction enzyme. The size of bands is labeled.

Table 2
Clinical characteristics and genetic variations of 10 patients with aHUS.

Patient		X1	AA1	CC1	DD1	FF1	GG1	HH1	111	JJ1	JJ2
Gender		M	M	M	M	F	F	M	M	M	M
Age of first episode		22y	9y	4y	6y	2y	5y	8m	28y	2y	70y
Period of follow-up		9y	21y	~1y	~1y	2y	~1y	1m	2y	34y	1y
Probable triggering events		URTI	URTI	URTI	Influenza A virus	None	Viral gastroenteritis	Surgery	Gastroenteritis	URTI	Surgery
C3 (mg/dl)[a]		55.9	110	123	111	110.8	67	40	109	58.5	NA
C4 (mg/dl)[a]		18.3	40.6	22	28	29.2	26	12.7	45	40.8	NA
CFH antigen (%)[a]		97	118	98	75	98	66	75	125	122	104
Hemolytic assay		+	–	–	–	–	+	–	+	±	±
Treatment		PE, HD, eculizumab	PE, HD	conservative	PE, HD	HD, PD	PE, FFP	HD, FFP	PE, HD, FFP	PE, HD	HD
Relapse (number)		1	5	0	0	0	0	0	1	7	1
Transplantation (number)		1	0	0	0	0	0	0	0	0	0
Outcome currently		ESRD	Complete remission[b]	Complete remission[b]	Complete remission[b]	ESRD	Complete remission[b]	Dead	ESRD	ESRD	ESRD
Missense mutations[c]											
CFH	c.184G>A	p.V62I	p.V62I	p.V62I		p.V62I	p.V62I		p.V62I		
	c.1204T>C[d]	p.Y402H	p.Y402H	p.Y402H	p.Y402H						
	c.2509G>A										p.V837I
	c.2808G>T	p.E936D				p.E936D	p.E936D	p.E936D(homo)	p.E936D	p.E936D	p.E936D
	c.3644G>A	**p.R1215Q**							**p.R1215Q**		
MCP	c.38C>T	p.S13F									
	c.565T>G				**p.Y189D**						
	c.1076C>T				**p.A359V**						
CFI	c.603A>C							p.R201S		p.R201S	p.R201S
	c.1217G>A				p.R406H						
C3	c.1273C>T							**p.R425C**			
	c.1685C>T					**p.S562L**					
	c.3470T>C		**p.I1157T**							**p.I1157T**	**p.I1157T**
CFB	c.94C>T									p.R32W	p.R32W(homo)
	c.95G>A	p.R32Q			p.R32Q				p.R32Q		
THBD	c.1418C>T	p.A473V	p.A473V(homo)	p.A473V	p.A473V		p.A473V	p.A473V	p.A473V		
	c.1499C>T								**p.T500M**		
CNV of CFH and CFHRs		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Abbreviations: y, year; m, month; CFH, complement factor H; MCP, membrane cofactor protein; CFI, complement factor I; C3, complement component 3; CFB, complement factor B; THBD, thrombomodulin; CFHRs, CFH related genes; URTI, upper respiratory tract infection; NA, not available; PE, plasma exchange; HD, hemodialysis; PD, peritoneal dialysis; FFP, fresh frozen plasma; ESRD, end-stage renal disease; CNV, copy number variation; homo, homozygote.

[a] Normal range: C3, 86–160 mg/dL; C4, 14–49 mg/dL; CFH, 50–150%.

[b] Complete remission is defined as normalization of both hematologic parameters (hematocrit > 30%; hemoglobin > 10 g/dL; lactate dehydrogenase < 460 U/L; platelet count > 150,000/μL) and renal function (serum creatinine < 1.3 mg/dL, equal to 114.92 μmol/L).

[c] Bold and underlined, definitely causative mutation; Bold, novel and potentially causative mutation; The A of the ATG of the initial Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1.

[d] Reference sequence of CFH (NM 000186.3) is c.1204C>T.

Table 3
Detailed characteristics of the causative or potentially causative mutations.

Gene	Mutation identified		Change in nucleotide	Domain	Location in 3D model	Prediction in silico[a]				Conservative[b]	Reported (Ref.)	Family	Genotype			
	Precursor	Mature				PolyPhen2	AGVGD	SIFT	PMut				Proband	Father	Mother	Brother
CFH	p.R1215Q	p.R1197Q	c.3644 G>A	SCR20	Exposed	Probably damaging	Likely interfere with function	Tolerated	Neutral	Yes	Reported (18,22)	X II	HE HE	HE WT	WT HE	WT 1:HE; 2: WT
C3	p.R425C	p.R403C	c.1273C>T	MG4	Exposed	Possible damaging	Most likely interfere with function	Damaging	Pathological	No	Novel	HH	HE	HE	WT	-
	p.S562L	p.S540L	c.1685C>T	MG6	Buried	Benign	Most likely interfere with function	Damaging	Neutral	No	Novel	FF	HE	WT	HE	HE
	p.I1157T	p.I1135T	c.3470 T>C	TED	Exposed	Benign	Most likely interfere with function	Tolerated	Neutral	Yes	Reported (17,19)	AA JJ	HE HE	HE HE	WT WT	– WT
MCP	p.Y189D	p.Y155D	c.565 T>G	SCR3	Buried	Probably damaging	Most likely interfere with function	Damaging	Neutral	Yes	Reported (20)	DD	HE	HE	WT	HE
	p.A359V	p.A325V	c.1076C>T	TM	–	Benign	Most likely interfere with function	Tolerated	Pathological	No	Novel	DD	HE	WT	HE	WT
THBD	p.T500M	p.T482M	c.1499C>T	STRD	–	Possible damaging	Most likely interfere with function	Tolerated	Pathological	Yes	Novel	II	HE	WT	HE	1: WT; 2:HE

Abbreviations: CFH, complement factor H; C3, complement component 3; MCP, membrane cofactor protein; THBD, thrombomodulin; SCR, short consensus repeat; MG, macroglobulin-like domain; TED, thioester-containing domain; TM, transmembrane region; STRD, serine and threonine-rich domain; HE, Heterozygote; WT, wild-type.

[a] The corresponding websites were described in the text.

[b] If more than 75% of the aligned species share the same amino acid, this amino acid is defined as conservative (i.e. yes).

3.6. Patient GG1

This female patient was the second of three children, but her elder sister was dead because of hemorrhagic shock at birth. Her father is Caucasian and her mother is Japanese. At 5 years of age, she presented with aHUS triggered by viral gastroenteritis with jaundice and ecchymosis on the trunk as the first manifestation. She received 12 plasma exchanges and methylprednisolone for 3 consecutive days, after which her laboratory tests were normal.

We did not identify a causative mutation or deletion of *CFH* or *CFH*-related genes (Table 2). But the CFH autoantibodies were detected by Western blot (lane 3 in Fig. 1). Both this patient and her unaffected father were positive in the hemolytic assay and the lysis activity was corrected by the addition of purified CFH (Table 2).

3.7. Patient HH1

This male patient was diagnosed with aHUS at 8 months of age, one month after his surgery for tetralogy of Fallot. After diagnosis, his condition deteriorated rapidly and he died within about 4 weeks despite being treated with fresh frozen plasma infusions and hemodialysis (Table 2).

He had a potentially causative mutation p.R425C in the β chain of *C3* (Tables 2 and 3, Fig. 2). His unaffected father had this mutation. His hemolytic activity was not enhanced.

3.8. Patient II1

This male patient had experienced several epileptic seizures in his teenage years. At 28 years of age, he developed HUS with extremely low platelet count (9×10^9/L) and rather severe renal dysfunction (creatinine, 13–14 mg/dL, equal to 1149–1238 μmol/L) (Table 2). His laboratory data were improved after treatment with fresh frozen plasma infusions for 1 day, 12 plasma exchanges and 4 weeks of hemodialysis. After discharge from the hospital 4 months later, he had a relapse. Renal biopsy revealed glomerular thrombotic microangiopathies. His renal function did not recover, although he was still being treated with hemodialysis at the most recent follow-up date.

He had the causative mutation p.R1215Q in *CFH* and one potentially causative mutation p.T500M in *THBD* (Tables 2 and 3, Fig. 2). His unaffected mother was a heterozygote for both mutations. Both he and his mother were positive in the hemolytic assay (Table 2).

3.9. Patients JJ1 and JJ2

Patient JJ1 was a male patient who developed aHUS at the age of 2. He then experienced 5 recurrences of aHUS before the age of 10 years (Table 2). At the age of 10, he was treated with peritoneal dialysis for acute renal failure. At 33 years of age, he again presented with HUS triggered with URTI. His laboratory data were improved after the 25th hemodialysis treatment. He had another recurrence of aHUS one year later. Treatments with 18 rounds of hemodialysis and plasma exchange were performed but the latter was interrupted because of anaphylactic shock. Patient JJ2, the father of patient JJ1, developed aHUS after his nephrectomy at 70 years of age. He was then treated with antiplatelet and antihypertensive agents, but 1 year and 3 months later, he developed acute renal failure with epileptic seizures and pulmonary edema. He was treated with hemodialysis at that time, but his renal function has been getting worse.

Both patients JJ1 and JJ2 carried the causative mutation p.I1157T in *C3* (Tables 2 and 3, Fig. 2). Both showed mildly elevated hemolytic activities (Table 2).

4. Discussion

In the present study, we identified 7 causative or potentially causative mutations in 8 of 10 Japanese patients with aHUS and the presence of CFH autoantibodies in another patient. Three of the mutations, p.R1215Q in *CFH*, p.I1157T in *C3*, and p.Y189D in *MCP*, were identified previously (Caprioli et al., 2006; Fremeaux-Bacchi et al., 2006; Maga et al., 2010; Martínez-Barricarte et al. 2008; Mukai et al., 2011), indicating that these mutations are causative for aHUS. The remaining 4 missense mutations, p.A359V in *MCP*, p.S562L and p.R425C in *C3*, and p.T500M in *THBD*, were novel. We considered them as potentially causative mutations based on the available information, including prediction programs, a search of the literature, and the position of the missense mutation in the three-dimensional structure, as described below. No causative mutations in *CFI* and *CFB* were detected and no genetic rearrangements in the RCA region were observed.

CFH, a principal regulator of the complement system, is composed of 20 SCRs. Several ligands, including C3b, C3d, heparin, and cell surface glycosaminoglycans, can bind to SCR19–20 in CFH (Manuelian et al., 2003). We identified the p.R1215Q mutation located in SCR20 of CFH in 2 aHUS patients who showed increased hemolytic activities. Functional analysis of a mutant CFH with p.R1215Q revealed reduced heparin-binding ability with a normal binding capacity for C3b, C3d, and the endothelial surface through glycosaminoglycans (Kajander et al., 2011; Morgan et al., 2011). This mutation has previously been reported in 3 Japanese aHUS patients in 2 families (Mukai et al., 2011). In the present study we identified it in 5 Japanese individuals, including 2 aHUS patients in 2 independent families. Therefore, the p.R1215Q mutation in *CFH* may be spread throughout the Japanese population.

C3 plays a major role in the complement system. In the present study, 5 aHUS patients carried 3 different missense mutations, p.R425C, p.S562L, and p.I1157T, in *C3*. Two mutations, p.R425C and p.S562L, are novel and the p.I1157T mutation has previously been reported in the United States and Spain (Maga et al., 2010; Martínez-Barricarte et al., 2008). The p.I1157T mutation was present in the thioester-containing domain, a hot area for C3 mutation. Mutagenesis studies revealed that the p.I1157A mutation in C3d attenuated the CFH19–20 binding by a factor of 4–6 when compared to wild-type C3d (Morgan et al., 2011). In addition, Ile1157 is an important contacting residue for complement receptor 2 (Clemenza and Isenman, 2000). Thus, we conclude that the p.I1157T mutation is causative. Two other novel mutations, p.R425C and p.S562L, are present in the macroglobulin 4 or 6 domain of the β chain in C3, respectively, and would be positioned on the surface of this domain based on the crystal structure (Janssen et al., 2005). More than two programs predicted that the p.R425C mutation was "Possibly damaging" or "Pathological" (Table 3). The p.S562L mutation occurred at the site close to the previously reported aHUS mutations, p.R592Q and p.R592W, which showed an impaired binding to the regulator protein, MCP (Fremeaux-Bacchi et al., 2008). Thus, we regarded them as potentially causative mutations.

MCP, a membrane-bound complement regulator highly expressed on most cell surfaces, acts as a cofactor for the CFI-mediated degradation of C3b and C4b (Lublin et al., 1988). The 4 extracellular SCRs are the binding site for C3b. Patient DD1 was a compound heterozygote for the p.Y189D and p.A359V mutations and developed aHUS after infection with influenza A-type virus, strongly indicating the precipitation of the hereditary and environmental risk factors for aHUS. In a French aHUS cohort, a heterozygous p.Y189D mutation was found in 3 out of 120 patients, 2 of whom were siblings (Fremeaux-Bacchi et al., 2006). The mutant MCP with the p.Y189D mutation led to a misfolded protein and an impaired function (Fremeaux-Bacchi et al., 2006). Therefore, we regarded p.Y189D as a causative mutation. The other mutation, p.A359V, was novel. This mutation occurred at the site close to the previously reported mutation, p.A353V (p.A304V in the previous reports), which has been identified in patients with aHUS and/or preeclampsia (Fang et al., 2008; Salmon et al., 2011). The p.A353V mutation had a defective ability to control the activation of the complement alternative pathway on a cell surface (Fang et al., 2008). In our study, only the proband carrying both the p.Y189D and p.A359V mutations developed aHUS, while the family members carrying only one of these mutations did not. The p.A359V mutation would modify the development of aHUS.

Mutations in thrombomodulin, a transmembrane endothelial glycoprotein encoded by *THBD*, accounted for the etiology in 3–5% of the aHUS patients (Delvaeye et al., 2009; Maga et al., 2010). The p.T500M mutation identified in patient II1 was located in the Ser- and Thr-rich region of thrombomodulin. Next to it, the p.P501L mutation was identified in an aHUS patient and exhibited defects in suppressing activation of the alternative complement pathway *in vitro* (Delvaeye et al., 2009). Moreover, three kinds of prediction in silico indicated that the p.T500M mutation was "Possibly damaging" or "Pathological" (Table 3). Considering this data together, we regarded this mutation as potentially causative and implicated in the pathogenesis of aHUS.

The CpG dinucleotide is a mutation hot spot and about 23% of single base-pair substitutions are CG→TG or CG→CA transitions, a frequency 5-fold higher than that for mutations in other dinucleotides (Krawczak et al., 1998). Among the 7 causative or potentially causative mutations, 4 mutations, p.R1215Q in *CFH*, p.S562L and p.R425C in *C3*, and p.T500M in *THBD*, occurred at the CpG dinucleotide.

Other synonymous and nonsynonymous SNPs were also identified in our patients (Table 2). Although these common variants are not extremely destructive, their pathogenic roles cannot be ignored, especially when combined (Heurich et al., 2011). The risk variant of *CFH* 402H weakened the CFH binding to sialylated surfaces (Herbert et al., 2007; Prosser et al., 2007), whereas the protective variant *CFH* 62I directly influenced the complement alternative pathway activity through a stronger binding to C3b and by acting as a better cofactor of CFI (Tortajada et al., 2009). The other protective variant *CFB* 32Q showed a reduction in C3bBb complex formation (Montes et al., 2009). Further "risk" combinations (*CFH* 62V/*CFB* 32R) resulted in a 2-fold increase in alterative pathway activation compared with the "protective" variants (*CFH* 62I/*CFB* 32Q) (Tortajada et al., 2009). All of the above-mentioned risk alleles were identified in our patients, half of whom were carriers of two or three "risk" alleles (Table 2). Therefore, the additive effects must dramatically exceed the effects of any single allele. A more comprehensive understanding of these disease-associated genetic variants is required.

Hemolytic assays are frequently used to evaluate the function of CFH (Heinen et al., 2006; Sanchez-Corral et al., 2004). Generally, the plasma samples containing the mutations in the C-terminal domains of CFH would show increased hemolytic activity. In our study, 2 aHUS patients with the *CFH* p.R1215Q mutation and the unaffected carriers in their families showed increased hemolytic activity, as did the other patient (GG1) with CFH autoantibodies.

Among the 7 patients carrying mutations in *CFH* or *C3* in the present study, one died and the remaining 5 patients progressed to end-stage renal disease. Patient AA1 obtained complete remission (Table 2). Five of 7 patients had a relapse. In contrast, one patient, DD1, the compound heterozygote for 2 mutations in *MCP*, had a better prognosis of complete remission without a relapse. These results obtained in Japanese aHUS patients were consistent with those obtained in Westerners (Loirat & Fremeaux-Bacchi, 2011). The overall midterm prognosis of aHUS is poor. At the first episode or within one year after onset, 50–70% or 60% of patients carrying the *CFH* or *C3* mutations, respectively, either died or reached

end-stage renal disease (Loirat & Fremeaux-Bacchi, 2011; Noris et al., 2010). Therefore, genetic information in patients with aHUS would be highly valuable for prognosis.

Incomplete penetrance of aHUS in the mutation carriers in the family has previously been reported (Caprioli et al., 2006; Loirat & Fremeaux-Bacchi, 2011; Noris et al., 2010). The present study confirmed this observation in Japanese aHUS patients (Fig. 2). The identified mutations were inherited from the patients' unaffected fathers or mothers. The single exception was a patient (JJ1) whose father (JJ2) had aHUS. None of the proband's siblings with the same mutation developed aHUS. It is likely that mutations do not directly cause an aHUS phenotype but rather modify the phenotype or predispose an individual to aHUS. The environmental factors and/or other genetic variations as a second hit are required for the manifestations of aHUS on one main genetic background (Francis et al., 2012; Pickering et al., 2007). Indeed, the onset of the disease was associated with infection or surgery in 9 out of 10 patients in our study (Table 2).

Patient X1 with the *CFH* p.R1215Q mutation received a live kidney transplantation, but failed three weeks later with recurrent aHUS. This result was consistent with the previous observations that the risk of post-transplant aHUS relapse is rather high in patients with *CFH* mutations (Loirat and Fremeaux-Bacchi, 2011; Noris & Remuzzi, 2009). In contrast, a lower risk of recurrence was reported in patients with the *MCP* mutation (Noris et al., 2010). One of the reasons for this variability is that CFH is a plasma protein synthesized by the liver, whereas MCP is synthesized by each cell locally. Therefore, combined liver–kidney transplantation might sometimes be a better option for CFH–associated patients based on a consideration of the risks/benefits in the individual patient (Saland et al., 2009). However, it should be noted that a relative kidney donor is not recommended, especially for patients with the *MCP* mutation, considering the possibly similar genetic background (Loirat and Fremeaux-Bacchi, 2011). Correspondingly, plasma exchange and plasma infusion may be a better and more logical choice for patients with the *CFH* mutation, but not efficient for correction of the essential defect in patients with MCP mutations, at least in theory.

In summary, the prevalence of genetic variation was evaluated in 10 Japanese aHUS patients. Seven causative or potentially causative mutations were identified in *CFH, C3, MCP*, and *THBD* in 8 patients and another patient was a carrier of CFH autoantibodies. The relationship between the genotype and phenotype was analyzed. Since the phenotype-genotype correlation of aHUS has clinical significance in predicting renal recovery and transplant outcome, a comprehensively accurate assessment of molecular variation would facilitate the clinical management for aHUS patients in Japan.

Conflict of interest

Dr. Fujimura is on the clinical advisory boards for Baxter Bioscience and Alexion Pharmaceuticals. Drs. Matsumoto, Hattori, and Ashida are on the clinical advisory board for Alexion Pharmaceuticals.

Contributions

T. Miyata and Y. Fujimura designed the study. X.P. Fan performed the genetic analysis with the guidance of S. Honda. Y. Yoshida and M. Matsumoto performed the protein analysis and hemolytic assay. Y. Yoshida, M. Matsumoto, Y. Sawada, M. Hattori, S. Hisanaga, R. Hiwa, F. Nakamura, M. Tomomori, S. Miyagawa, R. Fujimaru, H. Yamada, T. Sawai, Y. Ikeda, N. Iwata, O. Uemura, E. Matsukuma, Y. Aizawa, H. Harada, H. Wada, E. Ishikawa, A. Ashida, and M. Nangaku contributed to the sample collection, clinical data acquisition and interpretation of the data. X.P. Fan, T. Miyata, and Y. Fujimura interpreted the data and wrote the manuscript. All authors critically reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary Table 1

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2012.12.006.

References

Ariceta, G., Besbas, N., Johnson, S., Karpman, D., Landau, D., Licht, C., Loirat, C., Pecoraro, C., Taylor, C.M., Van de Kar, N., Vandewalle, J., Zimmerhackl, L.B., 2009. Guideline for the investigation and initial therapy of diarrhea-negative hemolytic uremic syndrome. Pediatric Nephrology 24, 687–696.

Boyce, T.G., Swerdlow, D.L., Griffin, P.M., 1995. Escherichia coli O157:H7 and the hemolytic-uremic syndrome. New England Journal of Medicine 333, 364–368.

Caprioli, J., Noris, M., Brioschi, S., Pianetti, G., Castelletti, F., Bettinaglio, P., Mele, C., Bresin, E., Cassis, L., Gamba, S., Porrati, F., Bucchioni, S., Monteferrante, G., Fang, C.J., Liszewski, M.K., Kavanagh, D., Atkinson, J.P., Remuzzi, G., 2006. Genetics of HUS: the impact of *MCPCFH*, and *IF* mutations on clinical presentation, response to treatment, and outcome. Blood 108, 1267–1279.

Clemenza, L., Isenman, D.E., 2000. Structure-guided identification of C3d residues essential for its binding to complement receptor 2 (CD21). Journal of Immunology 165, 3839–3848.

Delvaeye, M., Noris, M., De Vriese, A., Esmon, C.T., Esmon, N.L., Ferrell, G., Del-Favero, J., Plaisance, S., Claes, B., Lambrechts, D., Zoja, C., Remuzzi, G., Conway, E.M., 2009. Thrombomodulin mutations in atypical hemolytic-uremic syndrome. New England Journal of Medicine 361, 345–357.

Fang, C.J., Fremeaux-Bacchi, V., Liszewski, M.K., Pianetti, G., Noris, M., Goodship, T.H., Atkinson, J.P., 2008. Membrane cofactor protein mutations in atypical hemolytic uremic syndrome (aHUS), fatal Stx-HUS, C3 glomerulonephritis, and the HELLP syndrome. Blood 111, 624–632.

Francis, N.J., McNicholas, B., Awan, A., Waldron, M., Reddan, D., Sadlier, D., Kavanagh, D., Strain, L., Marchbank, K.J., Harris, C.L., Goodship, T.H., 2012. A novel hybrid *CFH/CFHR3* gene generated by a microhomology-mediated deletion in familial atypical hemolytic uremic syndrome. Blood 119, 591–601.

Fremeaux-Bacchi, V., Miller, E.C., Liszewski, M.K., Strain, L., Blouin, J., Brown, A.L., Moghal, N., Kaplan, B.S., Weiss, R.A., Lhotta, K., Kapur, G., Mattoo, T., Nivet, H., Wong, W., Gie, S., Hurault de Ligny, B., Fischbach, M., Gupta, R., Hauhart, R., Meunier, V., Loirat, C., Dragon-Durey, M.A., Fridman, W.H., Janssen, B.J., Goodship, T.H., Atkinson, J.P., 2008. Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. Blood 112, 4948–4952.

Fremeaux-Bacchi, V., Moulton, E.A., Kavanagh, D., Dragon-Durey, M.A., Blouin, J., Caudy, A., Arzouk, N., Cleper, R., Francois, M., Guest, G., Pourrat, J., Seligman, R., Fridman, W.H., Loirat, C., Atkinson, J.P., 2006. Genetic and functional analyses of membrane cofactor protein (CD46) mutations in atypical hemolytic uremic syndrome. Journal of the American Society of Nephrology 17, 2017–2025.

Goicoechea de Jorge, E., Harris, C.L., Esparza-Gordillo, J., Carreras, L., Arranz, E.A., Garrido, C.A., Lopez-Trascasa, M., Sanchez-Corral, P., Morgan, B.P., Rodriguez de Cordoba, S., 2007. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. Proceedings of the National Academy of Sciences of the United States of America 104, 240–245.

Heinen, S., Sanchez-Corral, P., Jackson, M.S., Strain, L., Goodship, J.A., Kemp, E.J., Skerka, C., Jokiranta, T.S., Meyers, K., Wagner, E., Robitaille, P., Esparza-Gordillo, J., Rodriguez de Cordoba, S., Zipfel, P.F., Goodship, T.H., 2006. De novo gene conversion in the RCA gene cluster (1q32) causes mutations in complement factor H associated with atypical hemolytic uremic syndrome. Human Mutation 27, 292–293.

Herbert, A.P., Deakin, J.A., Schmidt, C.Q., Blaum, B.S., Egan, C., Ferreira, V.P., Pangburn, M.K., Lyon, M., Uhrin, D., Barlow, P.N., 2007. Structure shows that a glycosaminoglycan and protein recognition site in factor H is perturbed by age-related

macular degeneration-linked single nucleotide polymorphism. Journal of the Biological Chemistry 282, 18960–18968.
Heurich, M., Martinez-Barricarte, R., Francis, N.J., Roberts, D.L., Rodriguez de Cordoba, S., Morgan, B.P., Harris, C.L., 2011. Common polymorphisms in C3, factor B, and factor H collaborate to determine systemic complement activity and disease risk. Proceedings of the National Academy of Sciences of the United States of America 108, 8761–8766.
Janssen, B.J., Huizinga, E.G., Raaijmakers, H.C., Roos, A., Daha, M.R., Nilsson-Ekdahl, K., Nilsson, B., Gros, P., 2005. Structures of complement component C3 provide insights into the function and evolution of immunity. Nature 437, 505–511.
Jozsi, M., Licht, C., Strobel, S., Zipfel, S.L., Richter, H., Heinen, S., Zipfel, P.F., Skerka, C., 2008. Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. Blood 111, 1512–1514.
Kajander, T., Lehtinen, M.J., Hyvarinen, S., Bhattacharjee, A., Leung, E., Isenman, D.E., Meri, S., Goldman, A., Jokiranta, T.S., 2011. Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement. Proceedings of the National Academy of Sciences of the United States of America 108, 2897–2902.
Kato, S., Matsumoto, M., Matsuyama, T., Isonishi, A., Hiura, H., Fujimura, Y., 2006. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. Transfusion 46, 1444–1452.
Krawczak, M., Ball, E.V., Cooper, D.N., 1998. Neighboring-nucleotide effects on the rates of germ-line single-base-pair substitution in human genes. American Journal of Human Genetics 63, 474–488.
Loirat, C., Fremeaux-Bacchi, V., 2011. Atypical hemolytic uremic syndrome. Orphanet Journal of Rare Diseases 6, 60.
Lublin, D.M., Liszewski, M.K., Post, T.W., Arce, M.A., Le Beau, M.M., Rebentisch, M.B., Lemons, L.S., Seya, T., Atkinson, J.P., 1988. Molecular cloning and chromosomal localization of human membrane cofactor protein (MCP). Evidence for inclusion in the multigene family of complement-regulatory proteins. Journal of Experimental Medicine 168, 181–194.
Maga, T.K., Nishimura, C.J., Weaver, A.E., Frees, K.L., Smith, R.J., 2010. Mutations in alternative pathway complement proteins in American patients with atypical hemolytic uremic syndrome. Human Mutation 31, E1445–E1460.
Manuelian, T., Hellwage, J., Meri, S., Caprioli, J., Noris, M., Heinen, S., Jozsi, M., Neumann, H.P., Remuzzi, G., Zipfel, P.F., 2003. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. Journal of Clinical Investigation 111, 1181–1190.
Martínez-Barricarte, R., Montes, T., Pinto, S., Sánchez-Corral, P., López-Trascasa, M., Morgan, B.P., Harris, C.L., Córdoba, S.R.d., Novel C3 mutations associated with atypical haemolytic uraemic syndrome. Poster presented at: XXII International Complement Workshop 2008. Sep. 28-Oct. 2, 2008; Basel, Switzerland.
Montes, T., Tortajada, A., Morgan, B.P., Rodriguez de Cordoba, S., Harris, C.L., 2009. Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B. Proceedings of the National Academy of Sciences of the United States of America 106, 4366–4371.
Moore, I., Strain, L., Pappworth, I., Kavanagh, D., Barlow, P.N., Herbert, A.P., Schmidt, C.Q., Staniforth, S.J., Holmes, L.V., Ward, R., Morgan, L., Goodship, T.H., Marchbank, K.J., 2010. Association of factor H autoantibodies with deletions of CFHR1, CFHR3, CFHR4, and with mutations in *CFHCFICD46*, and *C3* in patients with atypical hemolytic uremic syndrome. Blood 115, 379–387.
Morgan, H.P., Schmidt, C.Q., Guariento, M., Blaum, B.S., Gillespie, D., Herbert, A.P., Kavanagh, D., Mertens, H.D., Svergun, D.I., Johansson, C.M., Uhrin, D., Barlow, P.N., Hannan, J.P., 2011. Structural basis for engagement by complement factor H of C3b on a self surface. Nature Structural and Molecular Biology 18, 463–470.
Mukai, S., Hidaka, Y., Hirota-Kawadobora, M., Matsuda, K., Fujihara, N., Takezawa, Y., Kubota, S., Koike, K., Honda, T., Yamauchi, K., 2011. Factor H gene variants in Japanese: its relation to atypical hemolytic uremic syndrome. Molecular Immunology 49, 48–55.
Noris, M., Caprioli, J., Bresin, E., Mossali, C., Pianetti, G., Gamba, S., Daina, E., Fenili, C., Castelletti, F., Sorosina, A., Piras, R., Donadelli, R., Maranta, R., van der Meer, I., Conway, E.M., Zipfel, P.F., Goodship, T.H., Remuzzi, G., 2010. Relative role of genetic complement abnormalities in sporadic and familial aHUS and their impact on clinical phenotype. Clinical Journal of the American Society of Nephrology 5, 1844–1859.
Noris, M., Remuzzi, G., 2009. Atypical hemolytic-uremic syndrome. New England Journal of Medicine 361, 1676–1687.
Pickering, M.C., de Jorge, E.G., Martinez-Barricarte, R., Recalde, S., Garcia-Layana, A., Rose, K.L., Moss, J., Walport, M.J., Cook, H.T., de Cordoba, S.R., Botto, M., 2007. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. Journal of Experimental Medicine 204, 1249–1256.
Prosser, B.E., Johnson, S., Roversi, P., Herbert, A.P., Blaum, B.S., Tyrrell, J., Jowitt, T.A., Clark, S.J., Tarelli, E., Uhrin, D., Barlow, P.N., Sim, R.B., Day, A.J., Lea, S.M., 2007. Structural basis for complement factor H linked age-related macular degeneration. Journal of Experimental Medicine 204, 2277–2283.
Richards, A., Kemp, E.J., Liszewski, M.K., Goodship, J.A., Lampe, A.K., Decorte, R., Muslumanoglu, M.H., Kavukcu, S., Filler, G., Pirson, Y., Wen, L.S., Atkinson, J.P., Goodship, T.H., 2003. Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. Proceedings of the National Academy of Sciences of the United States of America 100, 12966–12971.
Roumenina, L.T., Loirat, C., Dragon-Durey, M.A., Halbwachs-Mecarelli, L., Sautes-Fridman, C., Fremeaux-Bacchi, V., 2011. Alternative complement pathway assessment in patients with atypical HUS. Journal of Immunological Methods 365, 8–26.
Saland, J.M., Shneider, B.L., Bromberg, J.S., Shi, P.A., Ward, S.C., Magid, M.S., Benchimol, C., Seikaly, M.G., Emre, S.H., Bresin, E., Remuzzi, G., 2009. Successful split liver-kidney transplant for factor H associated hemolytic uremic syndrome. Clinical Journal of the American Society of Nephrology 4, 201–206.
Salmon, J.E., Heuser, C., Triebwasser, M., Liszewski, M.K., Kavanagh, D., Roumenina, L., Branch, D.W., Goodship, T., Fremeaux-Bacchi, V., Atkinson, J.P., 2011. Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. PLoS Medicine 8, e1001013.
Sanchez-Corral, P., Gonzalez-Rubio, C., Rodriguez de Cordoba, S., Lopez-Trascasa, M., 2004. Functional analysis in serum from atypical Hemolytic Uremic Syndrome patients reveals impaired protection of host cells associated with mutations in factor H. Molecular Immunology 41, 81–84.
Sellier-Leclerc, A.L., Fremeaux-Bacchi, V., Dragon-Durey, M.A., Macher, M.A., Niaudet, P., Guest, G., Boudailliez, B., Bouissou, F., Deschenes, G., Gie, S., Tsimaratos, M., Fischbach, M., Morin, D., Nivet, H., Alberti, C., Loirat, C., 2007. Differential impact of complement mutations on clinical characteristics in atypical hemolytic uremic syndrome. Journal of the American Society of Nephrology 18, 2392–2400.
Sethi, S., Fervenza, F.C., 2012. Membranoproliferative glomerulonephritis–a new look at an old entity. New England Journal of Medicine 366, 1119–1131.
Skerka, C., Jozsi, M., Zipfel, P.F., Dragon-Durey, M.A., Fremeaux-Bacchi, V., 2009. Autoantibodies in haemolytic uraemic syndrome (HUS). Journal of Thrombosis and Haemostasis 101, 227–232.
Tarr, P.I., Gordon, C.A., Chandler, W.L., 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet 365, 1073–1086.
Tortajada, A., Montes, T., Martinez-Barricarte, R., Morgan, B.P., Harris, C.L., de Cordoba, S.R., 2009. The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. Human Molecular Genetics 18, 3452–3461.
Venables, J.P., Strain, L., Routledge, D., Bourn, D., Powell, H.M., Warwicker, P., Diaz-Torres, M.L., Sampson, A., Mead, P., Webb, M., Pirson, Y., Jackson, M.S., Hughes, A., Wood, K.M., Goodship, J.A., Goodship, T.H., 2006. Atypical haemolytic uraemic syndrome associated with a hybrid complement gene. PLoS Medicine 3, e431.
Zipfel, P.F., Edey, M., Heinen, S., Jozsi, M., Richter, H., Misselwitz, J., Hoppe, B., Routledge, D., Strain, L., Hughes, A.E., Goodship, J.A., Licht, C., Goodship, T.H., Skerka, C., 2007. Deletion of complement factor H-related genes *CFHR1* and *CFHR3* is associated with atypical hemolytic uremic syndrome. PLoS Genetics 3, e41.

Ticlopidine-associated ADAMTS13 activity deficient thrombotic thrombocytopenic purpura in 22 persons in Japan: a report from the Southern Network on Adverse Reactions (SONAR)

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disorder. The classic TTP 'pentad' is thrombocytopenia, microangiopathic hemolytic anemia (MAHA), renal impairment, neurological symptoms, and fever (Amorosi & Ultmann, 1966). Laboratory studies identified deficiency of plasma ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs 13) activity (ADAMTS13:AC) among some TTP patients (Furlan *et al*, 1998; Tsai & Lian, 1998). ADAMTS13 cleaves the peptide bond between Thy1605 and Met1606 in the A2 domain of von Willebrand factor (VWF) subunit. VWF is released into the plasma as unusually large VWF multimers (UL-VWFMs), which are degraded into smaller size VWF multimers by ADAMTS13. In the late 1990's, studies in the United States identified 117 cases of TTP that developed after initiation of the thienopyridine, ticlopidine; although at that time, ADAMTS13 activity levels were not widely available (Bennett *et al*, 1999; Steinhubl *et al*, 1999). A study of seven patients in the United States with ticlopidine-associated TTP found that all seven had severe deficiency of ADAMTS13 activity and five had detectable antibodies to ADAMTS13 activity (Tsai *et al*, 2000). We now report on 22 individuals from Japan with ticlopidine-induced TTP and compare these findings to those from the United States. Ticlopidine was the primary anti-platelet agent in Japan from 1989 to 2006.

Since 1998, our laboratory at Nara Medical University has been a nationwide referral centre in Japan for thrombotic microangiopathies (TMAs), including TTP (Fujimura & Matsumoto, 2010). The study protocol was approved by the Ethics Committee of Nara Medical University Hospital. TTP diagnostic criteria were: microangiopathic haemolytic anaemia (haemoglobin $\leq$120 g/l), Coombs test negative, undetectable serum haptoglobin (<1 μmol/l), more than 2 fragmented red cells (schistocytes) in a microscopic field with ×100 magnification, increased serum lactate dehydrogenase (LDH) above institutional baseline, thrombocytopenia (platelet count $\leq 100 \times 10^9$/l), absence of evidence for disseminated intravascular coagulation and no other identifiable cause of TTP. Additional information on fever $\geq$37°C; and central nervous system and renal function data were abstracted. Patients were included if, in addition to criteria for idiopathic TTP, the patient had received ticlopidine prior to TTP onset. Before therapeutic plasma exchange or plasma infusion was initiated, whole blood samples (five ml) were withdrawn from each patient and placed into plastic tubes containing 1/10 volume of 3·2% sodium citrate. Plasma was separated by centrifugation at 3000 g for 15 min at 4°C, kept in aliquots at −80°C until testing, and sent to our laboratory with clinical information. Until March 2005, ADAMTS13:AC was determined by classic VWF multimer (VWFM) assay with a detection limit of 3% of the normal control (Furlan *et al*, 1996; Kinoshita *et al*, 2001). Thereafter, a chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA) with a detection limit of 0·5% of the normal control was developed, and replaced the VWFM assay. Plasma ADAMTS13 inhibitor (ADAMTS13:INH) titres were analysed either by classic VWFM assay or chromogenic ADAMTS13-act-ELISA using heat-inactivated plasmas at 56°C for 30 min.

A total of 22 ticlopidine-associated TTP patients fulfilled the inclusion criteria (Table I). Age at diagnosis ranged from 41 to 89 years, with the median age of onset of 69 years. Females accounted for 45·5% of the cohort. Ticlopidine had been administered for a median of 27·5 d (range, 14–35 d) but was discontinued after a clinical diagnosis of TTP was made. Median values for hemoglobin were 83 (60–146) g/l, platelets 9·5 (3–57) $\times 10^9$/l, and serum creatinine 132·6 (35–380) μmol/l. Abnormal neurological findings were noted in 63·6%. All of the patients had <5% ADAMTS13:AC activity and detectable inhibitors to ADAMTS13 at the time of presentation. ADAMTS13:INH titres were 0·5 to <1·0 Bethesda units (BU)/ml in 4·5% of the patients, 1·0 to <2·0 BU/ml in 13·5%, 2·0 to <5·0 BU/ml in 45·5%, 5·0 to <10 BU/ml in 18·2%, and 4·5% of the patients had ADAMTS13:INH titres of $\geq$10 BU/ml. Mortality during the acute TTP episode was 9·0%. Mortality was highest among persons 60 years of age or older (10·0% vs. 0·0%). Therapeutic plasma exchange was performed in 72·7%, at a median of 3 d after the onset of TTP (range 1–5 d), and the TTP resolved at a median of 8 d (range 3–28 d). Among four patients whose TTP cleared after 20 or more days of therapeutic plasma exchange, ADAMTS13:INH titres were 2,4·4, 17, and 20 BU/ml. Among 12 patients whose TTP resolved with therapeutic plasma exchange at <20 d, none had ADAMTS13:INH titres >4 BU/ml. Both ticlopidine-associated TTP deaths did not receive therapeutic plasma exchange.

To our knowledge, this is the first study to report detailed characteristics of ticlopidine-associated TTP among patients outside of the United States. Our findings, from a cohort of

Table I. Characteristics of ticlopidine-associated thrombotic thrombocytopenic purpura in Japan and United States.

Source	This paper	Bennett *et al* (1999)	Tsai *et al* (2000)	Steinhubl *et al* (1999)
Number of patients	22	98	7	19
Country	Japan	US	US	US
Aetiology	Ticlopidine	Ticlopidine	Ticlopidine	Ticlopidine
% female	45·50%	46·6%	70·0%	30·0%
Median age (years)	69 (41–89)	64·2 (11·1 = SD)	57 (42–89)	62 (38–75)
Platelets <20 × 10^9/l	96·0% (23/24)	71·9%	100·0%	89·4%
Haemoglobin <90 g/l	72·7%	26,9%	42·3%	66·7%
Creatinine >221 μmol/l	18·1%	30·1%	NA	47·0%
Neurological abnormalities	63·6%	73·1%	70·0%	73·7%
Median days ticlopidine (range)	27·5 (14–36)	21 (7–112)	21 (14–56)	21 (14–28)
% with coronary stent	13·6%	42·3%	57·1%	100·0%
% with other Coronary artery disease indication	31·2%	0·0%	14·3%	0·0%
% stroke prevention	55·8%	57·7%	14·3%	0·0%
Survival	91·03%	84·9%	100·0%	78·9%
% Therapeutic plasma exchange (TPE)	63·6%	74·2%	100·0%	68·4%
Survival without TPE	75·0%	42·1%	—	33·3%
Survival with TPE	100·0%	81·7%	100·0%	100·0%
% with ADAMTS13 activity deficiency (<10%)	100·0%	Not available	83·3%	Not available
% with ADAMTS13 inhibitors	100·0%	Not available	100·0%	Not available

ticlopidine-associated TTP patients in Japan, identified severe ADAMTS13 deficiency and antibodies to ADAMTS13 in 100% of these 22 individuals. A decade earlier, severe ADAMTS13 deficiency was reported in 100% of seven patients with ticlopidine-associated TTP in the Uni ted States and antibodies to ADAMTS13 in five of these patients (Bennett *et al*, 1999; Tsai *et al*, 2000). While ticlopidine-induced TTP is undoubtedly a rare disease, it is reassuring that the original observations reported from the United States have been independently replicated in Japan (Bennett *et al*, 1999; Steinhubl *et al*, 1999).

Limitations of our study should be identified. Follow-up ended at the time of hospital discharge, which prevented us from reporting on relapse rates. Ticlopidine is rarely used today, having been replaced by clopidogrel in 1999 because of safety concerns. Our research has shown that clopidogrel, unlike ticlopidine, does not lead to ADAMTS13 antibody formation and deficiency of ADAMTS13 activity and the rare cases of clopidogrel-associated TTP are not responsive to therapeutic plasma exchange. Also, very little is known about TTP associated with prasugrel (the newest thienopyridine), despite 14 cases of prasugrel-associated TTP having been reported to the Food and Drug Administration in 2009 and 2010 (Jacob *et al*, 2012). Careful pharmacovigilance to identify severe adverse drug reactions developing among small numbers of persons can serve as important warning signals for potentially serious adverse drug events internationally.

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References

Amorosi, E.L. & Ultmann, J.E. (1966) Thrombotic thrombocytopenic purpura: report of 16 cases and review of the literature. *Medicine*, **45**, 139–159.

Bennett, C.L., Davidson, C.J., Raisch, D.W., Weinberg, P.D., Bennett, R.H. & Feldman, M.D. (1999) Thrombotic thrombocytopenic purpura associated with ticlopidine in the setting of coronary artery stents and stroke Prevention. *Archives of Internal Medicine*, **159**, 2524–2528.

Fujimura, Y. & Matsumoto, M. (2010) Registry of 919 patients with thrombotic microangiopathies across Japan: database of Nara Medical University during 1998–2008. *Internal Medicine*, **49**, 7–15.

Furlan, M., Robles, R. & Lammle, B. (1996) Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*, **87**, 4223–4234.

Furlan, M., Robles, R., Galbusera, M., Remuzzi, G., Kyrle, P.A., Brenner, B., Krause, M., Scharrer, I., Aumann, V., Mittler, U., Solenthaler, M. & Lämmle, B. (1998) von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *New England Journal of Medicine*, **339**, 1578–1584.

Jacob, S., Dunn, B.L., Qureshi, Z.P., Bandarenko, N., Kwaan, H.C., Pandey, D.K., McKoy, J.M., Barnato, S.E., Winters, J.L., Cursio, J.F., Weiss, I., Raife, T.J., Carey, P.M., Sarode, R., Kiss, J.E., Danielson, C., Ortel, T.L., Clark, W.F., Rock, G., Matsumoto, M., Fujimura, Y., Zheng, X.L., Chen, H.J., Chen, F., Armstrong, J.M., Raisch, D.W. & Bennett, C.L. (2012) Ticlopidine-, clopidogrel-, and prasugrel-associated thrombotic thrombocytopenic purpura: a twenty-year review. A report from the Southern Network on Adverse Reactions (SONAR). *Seminars in Thrombosis & Hemostasis*, **38**, 845–853.

Kinoshita, S., Yoshioka, A., Park, Y.D., Ishizashi, H., Konno, M., Funato, M., Matsui, T., Titani, K., Yagi, H., Matsumoto, M. & Fujimura, Y. (2001) Upshaw-Schulman syndrome revisited: a concept of congenital thrombotic thrombocytopenic purpura. *International Journal of Hematology*, **74**, 101–108.

Steinhubl, S.R., Tan, W.A., Foody, J.M. & Topol, E.J. (1999) Incidence and clinical course of thrombotic thrombocytopenic purpura due to ticlopidine following coronary stenting. EPISTENT investigators. Evaluation of platelet IIb/IIIa Inhibitor for stenting. *JAMA*, **281**, 806–810.

Tsai, H.M. & Lian, E.C. (1998) Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *New England Journal of Medicine*, **339**, 1585–1594.

Tsai, H.M., Rice, L., Sarode, R., Chow, T.W. & Moake, J.L. (2000) Antibody inhibitors to von Willebrand factor metalloproteinase and increased binding of von Willebrand factor to platelets in ticlopidine-associated thrombotic thrombocytopenic purpura. *Annals of Internal Medicine*, **132**, 794–799.

ADAMTS13 unbound to larger von Willebrand factor multimers in cryosupernatant: implications for selection of plasma preparations for thrombotic thrombocytopenic purpura treatment

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BACKGROUND: Thrombotic thrombocytopenic purpura (TTP) is characterized by deficient ADAMTS13 activity. Treatment involves plasma exchange (PE). Both fresh-frozen plasma (FFP) and cryosupernatant (CSP) are used, but it remains to be determined which is more effective.

STUDY DESIGN AND METHODS: To analyze the interaction between von Willebrand factor (VWF) and ADAMTS13, we used large-pore isoelectric focusing (IEF) analysis followed by detection with anti-ADAMTS13 monoclonal antibody. FFP, CSP, cryoprecipitate (CP), and purified ADAMTS13 were analyzed for their effects on high shear stress–induced platelet aggregation (H-SIPA).

RESULTS: IEF analysis of normal plasma revealed three groups of ADAMTS13 bands with pI of 4.9 to 5.6, 5.8 to 6.7, and 7.0 or 7.5. Two band groups (pI 4.9-5.6 and 5.8-6.7) were found in plasma of a patient with Type 3 von Willebrand disease, in which VWF is absent, whereas no bands were found in plasma of a patient with congenital ADAMTS13 deficiency. Mixing these plasmas generated the bands at pI 7.0 or 7.5, representing the VWF-ADAMTS13 complex; these bands were absent in CSP. FFP and purified ADAMTS13 down regulated H-SIPA in a dose-dependent manner. However, CP did not inhibit H-SIPA in the initial phase, and the degree of inhibition at the endpoint was almost indistinguishable from those of the other two plasma products.

CONCLUSION: Both plasma products (FFP and CSP) are effective for PE in TTP patients. However, CSP may be more favorable, because it has lower levels of VWF and almost normal ADAMTS13 activity, but lower levels of ADAMTS13 in complex with larger VWF multimers.

Von Willebrand factor (VWF), a multimeric hemostatic glycoprotein, is secreted from vascular endothelial cells into circulation as unusually large VWF multimers (UL-VWFMs).[1] The UL-VWFM is the most biologically active form with regard to platelet (PLT) adhesion properties.[2] Under conditions of high shear stress, UL-VWFMs cause enhanced PLT aggregation and give rise to VWF-rich thrombi in the microvasculature. ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13) down regulates the function of UL-VWFMs by cleaving the VWF A2 domain at the Tyr1605–Met1606 bond, yielding

ABBREVIATIONS: ADAMTS13 = a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13; CP = cryoprecipitate; CSP = cryosupernatant; dp = depleted; H-SIPA = high shear stress–induced platelet aggregation; IAA = iodoacetamide; IEF = isoelectric focusing; pd = plasma derived; PE = plasma exchange; T3-VWD = Type 3 von Willebrand disease; TBS = Tris-buffered saline; TTP = thrombotic thrombocytopenic purpura; UL-VWFM(s) = unusually large VWF multimer(s); USS = Upshaw-Schulman syndrome.

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a series of smaller molecular forms.[3-5] The proteolytic activity of ADAMTS13 is located in its amino-terminal metalloprotease domain, but optimal enzyme activity requires cooperative interactions with other domains of the ADAMTS13 molecule.[6]

Deficiency of ADAMTS13 activity causes a life-threatening generalized disease, thrombotic thrombocytopenic purpura (TTP), which can be caused either by mutation of the ADAMTS13 gene (Upshaw-Schulman syndrome [USS]) or by acquisition of autoantibodies against the ADAMTS13 enzyme.[7] USS is often treated by prophylactic infusions of fresh-frozen plasma (FFP) as a source of ADAMTS13, but cryoprecipitate (CP) has also been effective.[8,9] On the other hand, for patients with acquired TTP, plasma exchange (PE) is the first-line treatment.[10] For PE treatment, cryosupernatant (CSP) is preferentially used in Canada, but FFP is used in many other countries, including Japan. However, it has not been firmly established which material is more favorable for PE.[11] In this context, immunoprecipitation using anti-VWF revealed that approximately 3% of ADAMTS13 in plasma is bound to VWF, with a stoichiometry of one ADAMTS13 molecule to 250 VWF monomeric subunits.[12] However, the characteristics of the VWF-ADAMTS13 complex in the plasma milieu remain unclear, as does the physiologic relevance of functional differences, if any, between the bound and unbound forms of ADAMTS13.

To address these issues and analyze the VWF-ADAMTS13 complex in the plasma milieu, we employed isoelectric focusing (IEF) analysis using a large-pore agarose-acrylamide composite gel. Using this method, we were able to visualize the VWF-ADAMTS13 complex. We found that in the plasma milieu, ADAMTS13 forms a complex with larger VWFMs, but is less likely to do so with smaller VWFMs (dimers and tetramers); the complex can be separated from its unbound counterpart by cryoprecipitation. Based on these observations, we hypothesize that the bound and unbound forms of ADAMTS13 possess functional differences with respect to the microvascular condition of the patient. Furthermore, we evaluated the functional differences between ADAMTS13 in CSP and CP, by testing their inhibitory effects on high shear stress–induced PLT aggregation (H-SIPA).

MATERIALS AND METHODS

Plasma samples

Anticoagulated blood containing 1/10 volume of 3.8% sodium citrate was collected from normal individuals and from patient with either Type 3 von Willebrand disease (T3-VWD) or USS (Patient USS-EE4). The citrated plasmas were then separated by centrifugation and stored at –80°C until use. T3-VWD plasma had less than 3% of the normal control levels of both VWF antigen and ristocetin cofactor. The USS-EE4 patient had plasma levels of both ADAMTS13 activity and antigen less than 0.5 and 0.1% of the normal control, respectively; the ADAMTS13 gene mutation was identified as 2259delA/2259delA.[8] Informed consent was obtained from all subjects.

Preparation of CSP and CP

FFP was prepared at Nara Red Cross Blood Center and stored in inventory at –30°C. However, FFP preparations beyond 1 year for the inventory were provided to us. These FFP preparations were then kept frozen at –80°C in our institution. Outdated FFP was then thawed overnight at 4°C, followed by centrifugation at 7000 × *g* for 30 minutes at 4°C. After centrifugation, the CSP was separated and kept frozen in aliquots at –80°C. For analysis of ADAMTS13 activity and VWF antigen, the CP was dissolved in one-fifth volume of 20 mmol/L Tris-buffered saline (TBS, pH 7.4) without rinsing.

For H-SIPA, the CP was rinsed with cold TBS containing 0.38% Na_3-citrate, 2 mmol/L benzamidine-HCl, 20 mmol/L 6-amino-*n*-caproic acid, and 0.02% NaN_3 and then centrifuged at 4°C. This procedure was repeated twice. Ultimately, the CP was dissolved in 1/10 volume of TBS containing 0.38% Na_3-citrate and then stored in aliquots at –80°C.

Purified plasma VWF and ADAMTS13

Purification of plasma VWF was performed essentially as previously described:[13] cryoprecipitation of outdated pooled FFP collected from normal volunteers, removal of fibronectin by gelatin-agarose affinity chromatography, precipitation with 40% saturated $(NH_4)_2SO_4$, and finally purification by size-exclusion chromatography in Sepharose 4B gel. Fractions eluted in the anterior half of the void volume of the Sepharose 4B column were pooled; the resulting protein consisted of higher VWF multimers and migrated as a single 250-kDa band on a sodium dodecyl sulfate (SDS)-5% polyacrylamide gel under reducing conditions.[13] After dialysis against TBS, the purified plasma-derived (pd)-VWF was kept frozen in aliquots at –80°C until use.

Purification of plasma ADAMTS13 was achieved using anti-ADAMTS13 monoclonal antibody (MoAb) A10 (IgG2b-κ)–coupled beads as recently described.[14] The epitope of A10 resides on the disintegrin-like domain of ADAMTS13.[15] Briefly, the CSP was prepared essentially as described above, from outdated FFP in the presence of two protease inhibitors (2 mmol/L benzamidine-HCl and 20 mmol/L 6-amino-*n*-caproic acid) and 0.02% NaN_3. The CSP was then applied to an A10-coupled column at 4°C and washed extensively. The ADAMTS13 bound to the column was eluted in two steps, first with 10% dimethyl sulfoxide (DMSO) and then with 40% DMSO. The ADAMTS13 eluted with 40% DMSO was pooled and concentrated and then purified by size-exclusion chromatography on a Superdex HR10 column. The purified

pd-ADAMTS13 migrated on a SDS-5% polyacrylamide gel as a single 170-kDa band before reduction and a single 190-kDa band after reduction; specific activity was 300 units/mg.[14] One unit of ADAMTS13 activity was defined as the amount contained in 1 mL of pooled normal plasma.

Assays for ADAMTS13 and VWF

The ADAMTS13 activity and antigen were measured with a chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA)[16] and an in-house sandwich ELISA using two MoAbs, respectively.[17] The VWF antigen was determined with a sandwich ELISA using a rabbit polyclonal anti-human VWF antibody (Dako Cytomation, Kyoto, Japan).[18] A value of 100% of the ADAMTS13 activity and antigen were defined as the amount in the pooled normal human plasmas, which were prepared from a total of 40 normal volunteers, consisting of 10 individuals from each ABO blood group.

Preparation of ADAMTS13-depleted plasma

The ADAMTS13-depleted (dp) plasma was prepared from the whole FFP using an A10-agarose column equilibrated with TBS containing a cocktail of protease inhibitors (5 mmol/L benzamidine, 2 mmol/L phenylmethanesulfonyl fluoride, and 20 mmol/L 6-amino-*n*-caproic acid, each final concentration) at room temperature. Flowthrough fractions were monitored with ADAMTS13 activity and antigen; both the values indicated less than 0.5 and 0.1% of the normal, respectively and were dialyzed with TBS containing 0.38% Na_3-citrate and stored in aliquots at –80°C.

IEF using an agarose-acrylamide composite gel

IEF gel plate was assembled with two glass plates and 1-mm-thick plastic spacers. Four grams of sucrose and 0.3 g of agarose (final 0.75%, agarose IEF, GE Healthcare Bio-Science AB, Uppsala, Sweden) were mixed with 34.2 mL of distilled water. The mixture was dissolved by microwave oven and kept at 56°C. Then, 1.67 mL of 30% acrylamide-bisacrylamide (final 1.25%), 1.67 mL of distilled water, 2.5 mL of 40% carrier ampholyte (Pharmalyte 3-10, GE Healthcare Bio-Science AB), 0.27 mL of ammonium peroxodisulfate (22.8 mg/mL), and 0.01 mL of *N,N,N′,N′*-tetramethylethylenediamine were added to this mixture. The mixture was poured into the IEF gel plate quickly and left for more than 1 hour at room temperature followed by 4°C overnight.

The IEF gel was placed on the Multiphor apparatus (GE Healthcare Bio-Science AB) equilibrated at 10°C. The electrode strips were prepared using 0.5 mol/L acetic acid at the anode and 0.5 mol/L sodium hydroxide at the cathode. The electrical conditions used for IEF were the first 30 minutes at a maximum of 100 V, 5 mA, and 15 W; then 60 minutes at a maximum of 200 V, 10 mA, and 6 W; and finally 90 minutes at a maximum of 1500 V, 15 mA, and 6 W. After IEF, the isolated proteins were electrophoretically transferred to nitrocellulose membrane.

Iodoacetamide effect on complex of ADAMTS13 and VWF in plasma milieu

Recent studies have indicated that free thiols exposed in ADAMTS13 play an important role to regulate thiol-disulfide exchange of VWF under a high shear stress. Furthermore, blocking these active thiols decreases ADAMTS13 activity in cleaving UL-VWFM under flow conditions.[19] We evaluated the effect of iodoacetamide (IAA), which blocks the free thiols and prevents the formation of a covalent complex through disulfide bonds. For this experiment, each reagent of ADAMTS13-dp plasma, purified pd-ADAMTS13, and pd-VWF was separately treated with or without 100 mmol/L IAA before mixing for 30 minutes at room temperature. The mixture of these three reagents was exposed to a high shear stress generated by a vortex mixer at 3200 rpm for 5 minutes. The final concentration of each reagent in this mixture (a total of 130 μL) was 60 μg/mL for pd-VWF, 2.3 μg/mL for purified pd-ADAMTS13, and 65 μL for ADAMTS13-dp plasma.

Two-dimensional gel electrophoresis using either polyacrylamide gel electrophoresis or agarose

In some experiments, after IEF the two-dimensional gel electrophoresis was performed using either SDS-5% polyacrylamide gel electrophoresis (PAGE) under reducing conditions or SDS-0.9% agarose gel electrophoresis under nonreducing conditions. The former was used for an analysis of ADAMTS13 antigen and the latter for VWF multimer patterns. In both instances, the separated proteins were electrophoretically transferred to polyvinylidene fluoride membrane or nitrocellulose membrane, and then the blot proteins were immunoreacted with anti-ADAMTS13 MoAb (WH2-11-1, an epitope residing on the fourth thrombospondin Type 1 domain of ADAMTS13)[20] or rabbit polyclonal anti-human VWF antibody and then visualized by chemiluminescent detection kits (Perkin-Elmer Life Science, Inc., Boston, MA).

H-SIPA in the absence of ADAMTS13

To reproduce PLT aggregation assumed to be occurring in TTP patients, H-SIPA at a constant shear rate of 108 dynes/cm^2 was measured with an argon laser-assisted cone PLT aggregometer (Toray Medical, Tokyo, Japan),[21] using a mixture of normal washed PLTs (300×10^9/L, final), ADAMTS13-dp plasma (29% vol/vol, final), and the purified pd-VWF (250% of the normal plasma, final).

For this assay, normal washed PLTs were prepared and suspended in a Hepes-Tyrode buffer (pH 7.3) containing 1.8 mmol/L $CaCl_2$.[22] The mixture with a total volume of

400 μL was preincubated at 37°C for 5 minutes, and then H-SIPA was measured for 6 minutes. The maximum PLT aggregation was seen in the absence of any additives, and the minimum or nonspecific PLT aggregation was determined in the presence of anti-VWF MoAb NMC-4 (10 μg IgG/mL, final), which totally blocks the VWF binding to PLT GPIb.[13]

For assessment of the inhibitory effect of various forms of pd-ADAMTS13 to H-SIPA, they were spiked into the above-mentioned assay mixtures and incubated for 5 minutes at 37°C before measurement. H-SIPA was measured at room temperature and completed within 2.5 hours after blood collection. The inhibition rate of H-SIPA was calculated in the following formula: Inhibition rate (%) = [1 − (% light transmittance of tested sample/% light transmittance of control)] × 100. These data were expressed as the mean ± SD. We calculated the inhibition rate in two points at 140 and 340 seconds after the initiation of H-SIPA. Comparison between these two points was tested for significance using paired t test using computer software (StatView, SAS Institute, Inc., Cary, NC). A p value of less than 0.05 was considered significant.

RESULTS

ADAMTS13 and VWF on IEF agarose-acrylamide composite gels

We detected the purified pd-ADAMTS13 (15 ng) as one band at pI 4.9 to 5.6 (median, 5.4) using anti-ADAMTS13 MoAb (WH2-11-1) on IEF agarose-acrylamide composite gels (Fig. 1, left panel). Next, we analyzed various amounts (1-10 μL) of normal citrated plasma and found that ADAMTS13 antigen in the plasma milieu could also be detected as a major band at pI 4.9 to 5.6, as in the case of purified pd-ADAMTS13. In plasma, however, two additional bands of ADAMTS13 antigen were also detected: one was composed of a cluster of blurred bands at pI 5.8 to 6.7, and the other consisted of two clear bands at pI 7.0 or 7.5. In T3-VWD plasma, two groups of ADAMTS13 bands, pI 4.9 to 5.6 and 5.8 to 6.7, were detected, but the bands at pI 7.0 or 7.5 were totally absent (Fig. 1, right panel). T3-VWD plasma lacks VWF antigen; therefore, the two groups of bands at pI 4.9 to 5.6 and 5.8 to 6.7 appear to exist independently of the presence of plasma VWF. Conversely, we assumed that the bands at pI 7.0 or 7.5 represented a complex with VWF that exists within the plasma milieu. The bands at pI 7.0 or 7.5 were also detected after mixing FFP with 1 mol/L NaCl (final), excluding the possibility that the complex is formed by an ionic linkage (data not shown).

Generation of the pI 7.0 of 7.5 band of ADAMTS13 complex with VWF

Next, we performed the mixing experiments shown in Fig. 2A. T3-VWD plasma spiked with purified pd-VWF yielded a new band at pI 7.5. USS-EE4 plasma initially

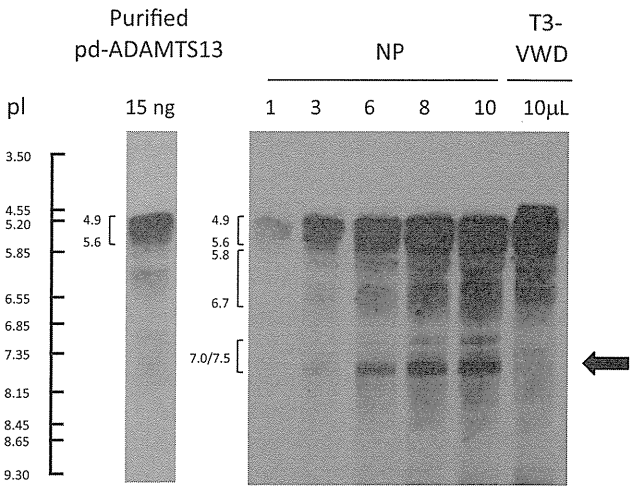

Fig. 1. Separation of plasma ADAMTS13 by IEF in healthy control and patient with T3-VWD. Purified pd-ADAMTS13, normal plasma (NP), and T3-VWD plasma were subjected to IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1). Purified pd-ADAMTS13 (15 ng) was detected as one band at pI 4.9 to 5.6 (median, 5.4; left panel). In various amounts (1-10 μL) of NP, ADAMTS13 antigen was detected as a major band at pI 4.9 to 5.6. Two additional groups of bands of ADAMTS13 antigen were also detected: pI 5.8 to 6.7 and pI 7.0 or 7.5. In T3-VWD, the ADAMTS13 band groups of pI 4.9 to 5.6 and 5.8 to 6.7 were detected, but the band of pI 7.0 or 7.5 was barely detectable (right panel). Arrow indicates the VWF-ADAMTS13 complex.

lacked three groups of ADAMTS13 bands (pI 4.9-5.6, 5.8-6.7, and 7.0/7.5), but once that plasma was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When T3-VWD and USS-EE4 plasma samples were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTS13.

ADAMTS13 (pI 7.5) is a noncovalent complex with VWF in the plasma milieu

We next evaluated the effects of IAA, which blocks free thiols and prevents the formation of disulfide bond–mediated covalent complexes, under high shear stress in a vortex mixer. As shown in Fig. 2B, the band at pI 7.5, representing the VWF-ADAMTS13 complex, was generated irrespective of the presence of IAA. When pd-VWF was spiked into this mixture, the density of the band at pI 7.5 increased. These results indicate that in our experiments, formation of the VWF-ADAMTS13 complex does not depend upon disulfide bond bridges.

ADAMTS13 is present in plasma in complex with a large VWFM

As shown in Fig. 3, IEF gel analysis of normal plasma revealed ADAMTS13 as three groups of bands (pI 4.9-5.6,

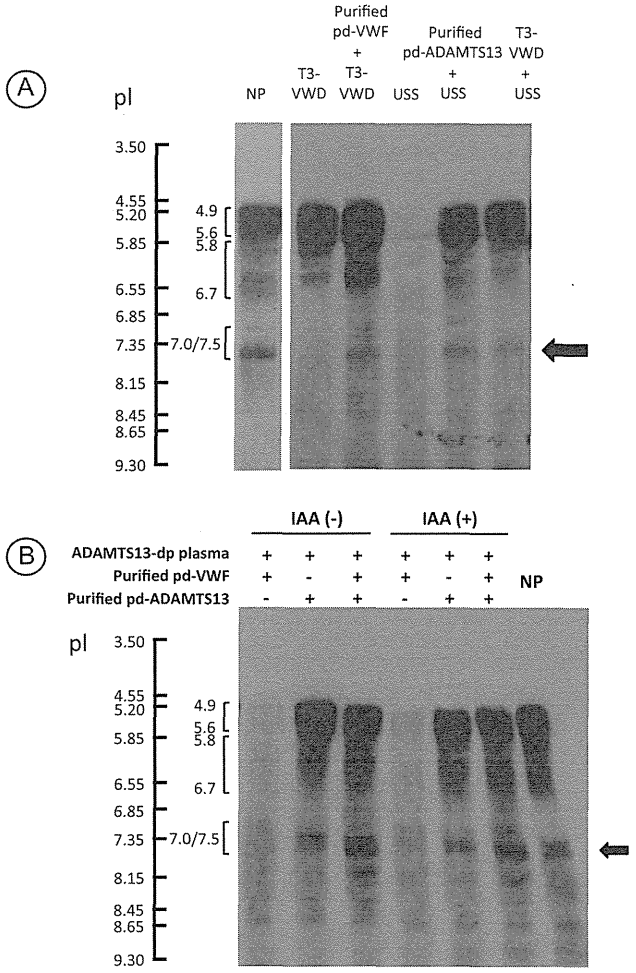

Fig. 2. Spiking experiments and effect of IAA treatment on VWF-ADAMTS13 complex formation. (A) VWF-ADAMTS13 complex formation was analyzed by IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1). Plasma of T3-VWD spiked with the purified pd-VWF generated a new band at pI 7.5. When plasma from a patient with USS was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When plasmas from T3-VWD and USS patients were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTS13. (B) Purified pd-VWF (3 μg), pd-ADAMTS13 (200 ng), and ADAMTS13-dp plasma were treated for 30 minutes at room temperature with 100 mmol/L IAA. ADAMTS13-dp plasma was mixed with purified pd-VWF (final concentration, 60 μg/mL) and/or pd-ADAMTS13 (final concentration, 2.3 μg/mL). Mixtures were exposed to high shear stress in a vortex mixer at maximum speed (3200 rpm) for 5 minutes. The VWF-ADAMTS13 complex, represented by the band at pI 7.5, is generated irrespective of the presence of IAA. Purified pd-VWF spiked into this mixture increased the density of this band. Arrow indicates the VWF-ADAMTS13 complex.

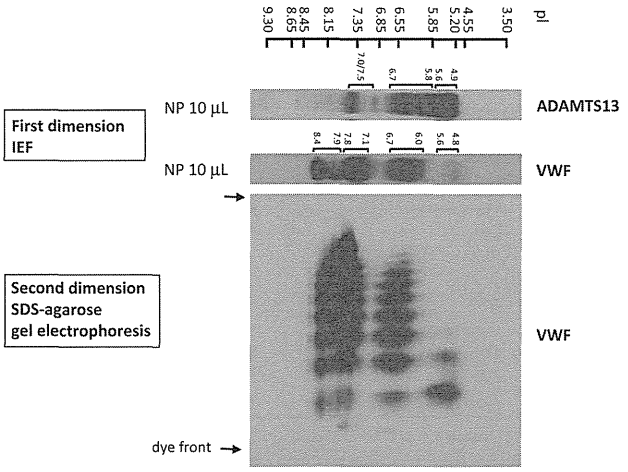

Fig. 3. Two-dimensional analysis of VWF in NP. Ten microliters of NP was subjected to IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1; upper panel) and polyclonal anti-VWF antibody (middle panel). IEF gel of NP was subjected to a second dimension of electrophoresis on a SDS-0.9% agarose gel and immunoblotting with anti-VWF polyclonal antibody. In the lower panel, the upper arrowhead indicates the start point of two-dimensional SDS-0.9% agarose gel electrophoresis; the lower arrowhead indicates the position of the dye front at the termination of electrophoresis. VWF antigen in NP was separated into four series of pI bands (4.8-5.6, 6.0-6.7, 7.1-7.8, and 7.9-8.4) by IEF (middle panel), whereas ADAMTS13 antigen was separated into three series of pI bands (4.9-5.6, 5.8-6.7, and 7.0/7.5; upper panel). Two-dimensional analysis of normal plasma by IEF followed by SDS-0.9% agarose gel electrophoresis (lower panel) revealed that plasma ADAMTS13 is primarily in complex with larger VWF multimers and to a lesser extent with smaller VWF multimers.

5.8-6.7, and 7.0/7.5; also see Fig. 1), and VWF antigen was largely separated into four series of bands: pI 4.8 to 5.6 (trace), 6.0 to 6.7, 7.1 to 7.8, and 7.9 to 8.4 (Fig. 3, middle).

Since two ADAMTS13 bands with pI 4.9 to 5.6 and 5.8 to 6.7 were seen in T3-VWD plasma (Fig. 1), both bands appeared to be present in plasma irrespective of the presence of VWF. Further, two-dimensional analysis of normal plasma (IEF gel followed by SDS-0.9% agarose gel electrophoresis) confirmed that ADAMTS13 forms a complex with a larger VWFM with pI 7.1 to 7.8, but less likely with a smaller VWFM (dimers and tetramers) with pI 4.8 to 5.6 (Fig. 3, lower panel).

Amounts of ADAMTS13 and VWF in CP and CSP

Gill and colleagues[23] reported that the level of VWF antigen in plasmas from normal individuals with blood group O is significantly lower than that in plasmas with non-O blood groups. Further, Feys and colleagues[12] indicated that ADAMTS13 is bound to VWF with a stoichiometry of one ADAMTS13 molecule to 250 VWF

TABLE 1. ADAMTS13 activity and VWF antigen levels in plasma products*

Plasma products	All (n = 120)	Blood type A (n = 30)	O (n = 30)	B (n = 30)	AB (n = 30)	Overall p value
ADAMTS13 activity (%)						
FFP	81 ± 16	84 ± 17	77 ± 15	80 ± 16	83 ± 13	NS
CSP	71 ± 14	72 ± 14	69 ± 17	70 ± 12	72 ± 13	NS
Recovery (%)†	92.7 ± 3.7	92.9 ± 3.6	95.2 ± 2.1	91.4 ± 3.9	91.2 ± 3.5	<0.01[a]
CP	5.6 ± 2.8	5.4 ± 2.4	3.5 ± 1.6	6.5 ± 2.9	6.9 ± 2.8	<0.01[a]
Recovery (%)†	7.3 ± 3.7	7.1 ± 3.6	4.8 ± 2.1	8.6 ± 3.9	8.8 ± 3.5	<0.01[a]
VWF antigen (%)						
FFP	124 ± 46	121 ± 49	80 ± 24	144 ± 32	150 ± 38	<0.01[b]
CSP	16 ± 7	15 ± 7	11 ± 3	19 ± 5	19 ± 6	<0.01[c]
Recovery (%)†	14.0 ± 2.6	12.7 ± 2.5	14.6 ± 2.2	14.6 ± 2.9	14.2 ± 2.4	<0.05[d]
CP	98 ± 35	100 ± 37	64 ± 18	112 ± 22	116 ± 32	<0.01[e]
Recovery (%)†	86.0 ± 2.6	87.3 ± 2.5	85.4 ± 2.2	85.4 ± 2.9	85.8 ± 2.4	<0.05[d]

* Overall p values were calculated using the Kruskal-Wallis H test. Significant differences between four groups (overall $p < 0.05$) were further analyzed by Mann-Whitney U test with Bonferroni correction.
† Recovery was calculated as the level in CP or CSP divided by the total (sum of levels in CP and CSP).
[a] $p < 0.01$ between O and B, AB, <0.05 between O and A.
[b] $p < 0.01$ between O and A, B, AB, <0.05 between A and B.
[c] $p < 0.01$ between O and A, B, AB, <0.01 between A and B, <0.05 between A and AB.
[d] $p < 0.05$ between A and O, B, AB.
[e] $p < 0.01$ between O and A, B, AB.
NS = no significant difference ($p \geq 0.05$).

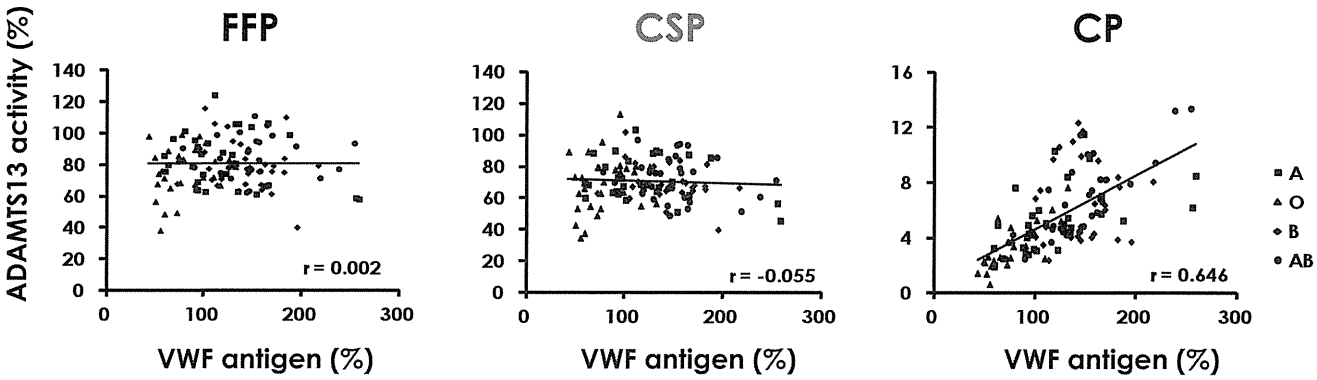

Fig. 4. Correlation between VWF antigen and ADAMTS13 activity in FFP, CSP, and CP. The correlation between ADAMTS13 activity and VWF antigen in FFP, CSP, and CP was analyzed. In FFP and CSP, there is no correlation between ADAMTS13 activity and VWF antigen. On the other hand, a significant correlation was observed between the two variables in CP ($r = 0.646$, $p < 0.01$).

monomeric subunits. Taking these two reports together, it is conceivable that the amount of a complex of ADAMTS13 and VWF in CP could be influenced by the ABO blood groups.

We analyzed ADAMTS13 activity and VWF antigen in FFP, CSP, and CP from 120 normal volunteers, with 30 individuals of each ABO blood type (A, B, O, and AB). The recovery rates of ADAMTS13 activity and VWF antigen in CP or CSP were expressed as the level in CP or CSP divided by the sum of the levels in both (CP + CSP). As summarized in Table 1, a mean of 7.3% (range, 4.8%-8.8%) of plasma ADAMTS13 activity was recovered in CP, whereas a mean of 92.7% (range, 91.2%-95.2%) remained in CSP. The amounts of ADAMTS13 remaining in CP from A, O, B, and AB plasmas were 5.4 ± 2.4, 3.5 ± 1.6, 6.5 ± 2.9, and 6.9 ± 2.8%, respectively; the amount of ADAMTS13 activity in CP was significantly lower in blood group O than in other blood groups. On the other hand, a mean of 86.0% (range, 85.4%-87.3%) of plasma VWF antigen was recovered in CP, whereas a mean of 14.0% (range, 12.7%-14.6%) remained in CSP. The amounts of VWF antigen in FFP, CP, and CSP from blood group O were significantly lower than in samples from other blood groups. The recovery rate of VWF antigen in CP was significantly higher in blood group A than in other blood groups.

As shown in Fig. 4, we analyzed the correlation between the levels of ADAMTS13 activity and VWF antigen in three plasma preparations with the Spearman rank test. We did not find a significant correlation between VWF antigen in FFP and ADAMTS13 activity in either FFP or CSP. In contrast, we did observe a significant correlation between ADAMTS13 activity in CP and VWF antigen in