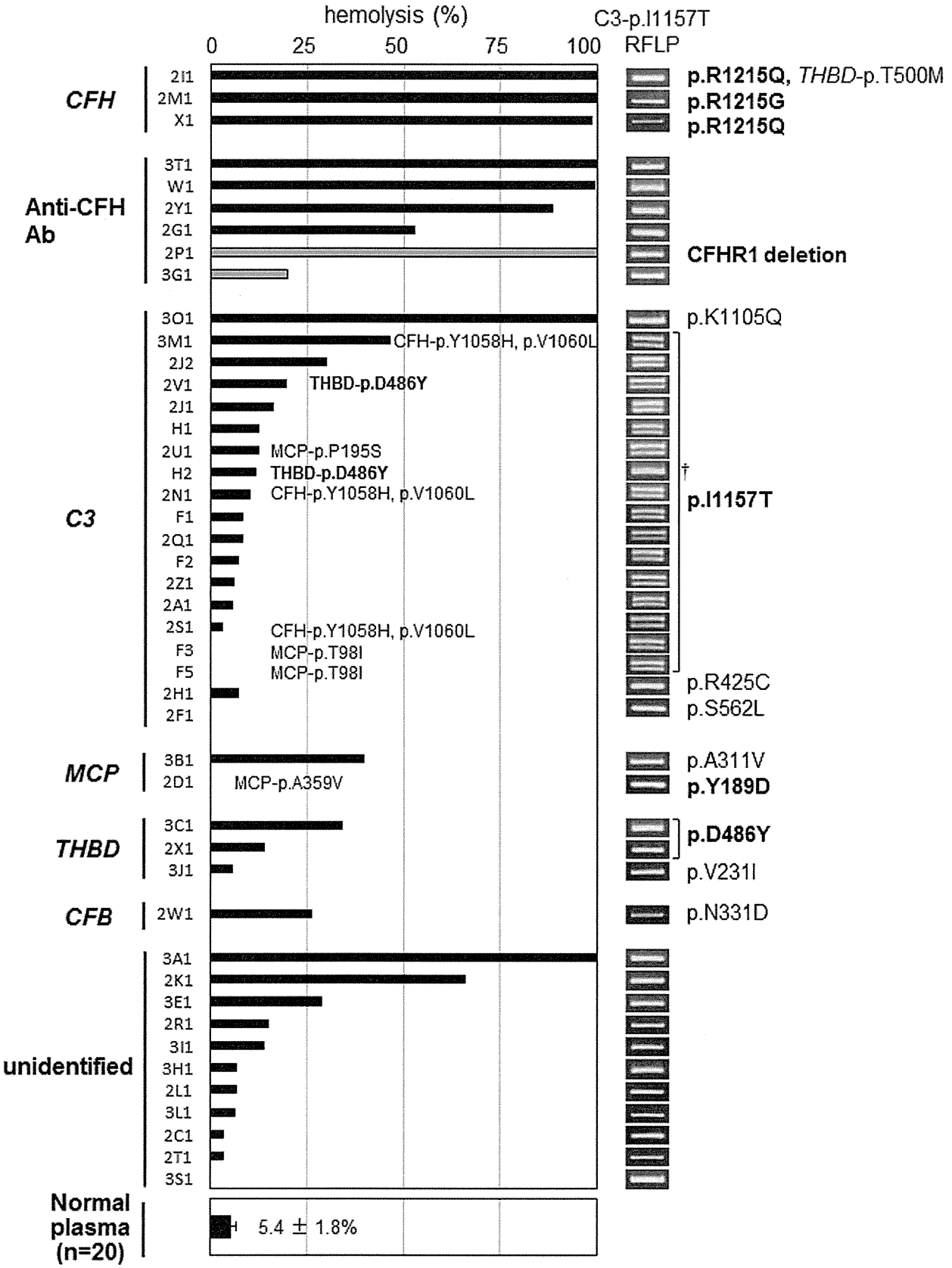
hemolysis (%)
0
25
50
75
100
C3-p.I1157T RFLP
CFH
2I1
2M1
X1
p.R1215Q, THBD-p.T500M
p.R1215G
p.R1215Q
Anti-CFH Ab
3T1
W1
2Y1
2G1
2P1
3G1
CFHR1 deletion
C3
3O1
3M1
CFH-p.Y1058H, p.V1060L
2J2
2V1
THBD-p.D486Y
2J1
H1
2U1
MCP-p.P195S
H2
THBD-p.D486Y
2N1
CFH-p.Y1058H, p.V1060L
F1
2Q1
F2
2Z1
2A1
2S1
CFH-p.Y1058H, p.V1060L
F3
MCP-p.T98I
F5
MCP-p.T98I
2H1
2F1
p.K1105Q
†
p.I1157T
p.R425C
p.S562L
MCP
3B1
2D1
MCP-p.A359V
p.A311V
p.Y189D
THBD
3C1
2X1
3J1
p.D486Y
p.V231I
CFB
2W1
p.N331D
unidentified
3A1
2K1
3E1
2R1
3I1
3H1
2L1
3L1
2C1
2T1
3S1
Normal plasma (n=20)
5.4 ± 1.8%

Fig 3. Relationship between hemolytic activity and complement abnormalities in 45 patients with atypical hemolytic uremic syndrome (aHUS). The results of the hemolytic assay for 45 aHUS patients are shown according to the predisposing (bold type) or potentially predisposing mutations and/or the acquired abnormalities. The patients carrying two mutations were classified according to the predisposing mutation, and the other mutations were described alongside the predisposing mutations or black bars. Two patients (2V1 and H2) were categorized into the C3 group, although they had two predisposing mutations (C3-p.I1157T and THBD-p.D486Y). In two patients (2P1 and 3G1), the hemolysis induced by serum was represented by gray bars. The normal range of hemolysis (5.4±1.8%: mean ± standard deviation) obtained from 20 healthy individuals (10 males and 10 females) is shown at the bottom of Fig 3. The results of the restriction fragment length polymorphism (RFLP) analysis of C3-p.I1157T are summarized to the right of the hemolytic assay results. Regarding the RFLP analysis of C3-p.I1157T (c.3470T>C), the heterozygous mutation (T/C) showed two bands (278 bp, 314 bp) and the homozygous mutation (C/C) showed one band (314 bp). † H2 had a homozygous mutation in C3-p.I1157T.

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no appreciable hemolysis. The enhanced hemolysis detected in these four family members was corrected by the addition of purified CFH (Fig 6C). However, none of the family members had anti-CFH autoantibodies or CFH deficiency. These results suggest that the family of Patient

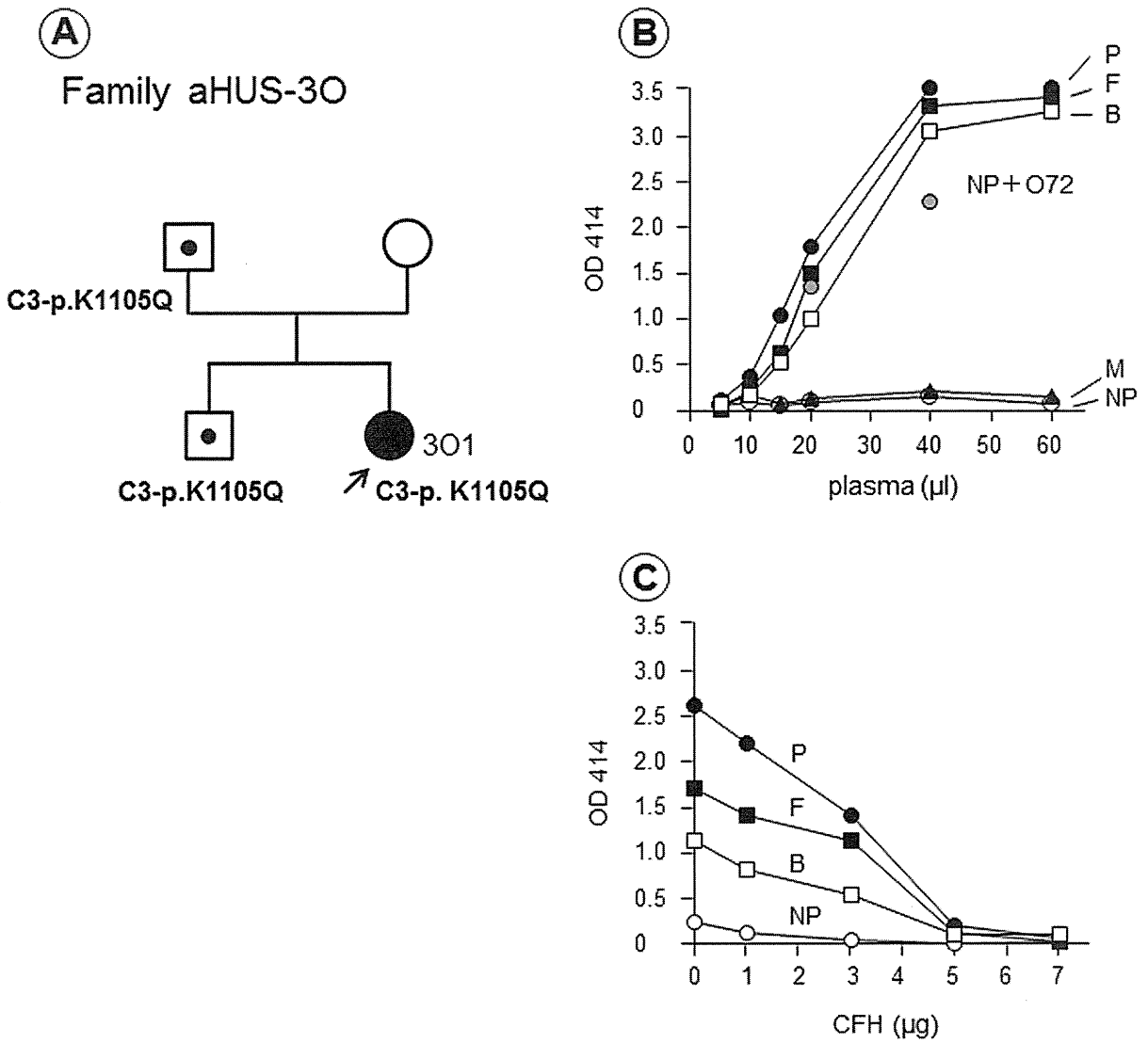

Fig 4. Family 3O with a C3-p.K1105Q mutation. (A) Patient 3O1 (female) developed atypical hemolytic uremic syndrome (aHUS) at the age of five months. (B) In the hemolytic assay, plasma samples from three family members (patient: P, father: F, and brother: B) induced strong hemolysis of sheep red blood cells (RBCs), but her mother (M) had no appreciable hemolysis, with results similar to normal plasma (NP). (C) Lysis of sheep RBCs detected in the three family members was corrected by the addition of complement factor H (CFH) in a dose-dependent manner. Genetic analysis showed that the patient and her father and brother, whose plasma clearly showed hemolysis, carried a potentially predisposing mutation p.K1105Q in C3, not in CFH.

doi:10.1371/journal.pone.0124655.g004

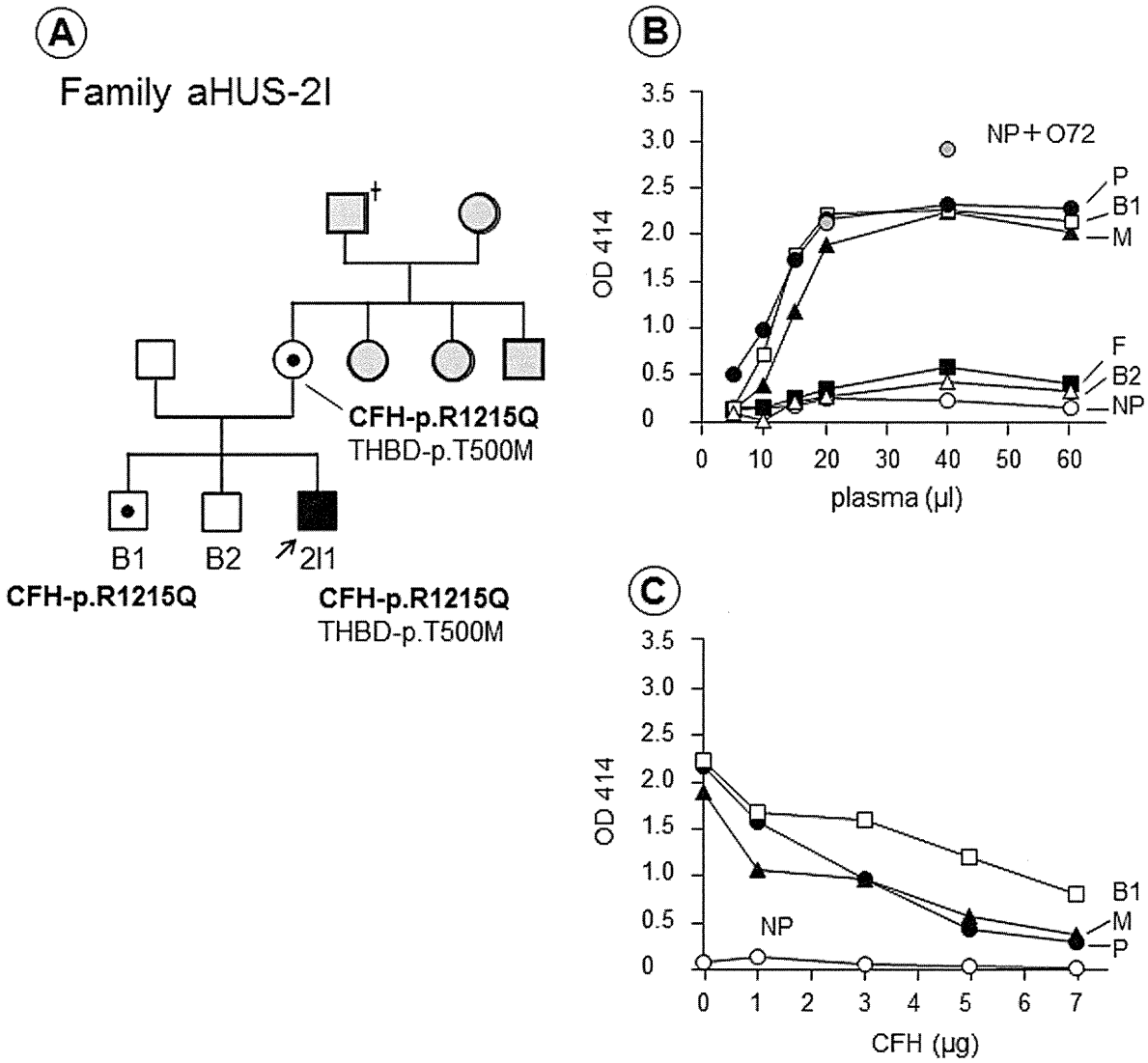

Fig 5. Family 2I with a CFH-p.R1215Q mutation. (A) Patient 2I1 (male) had episodes of atypical hemolytic uremic syndrome (aHUS) at the age of 28 and 29. Gray squares or circles indicate individuals who were not analyzed in this study. (B) The hemolytic assay showed that plasma from the patient (P), his mother (M), and one brother (B1) induced severe hemolysis, whereas samples from his father (F) and the other brother (B2) showed no appreciable hemolysis. (C) The hemolysis detected in the three family members was corrected by the addition of purified complement factor H (CFH). Genetic analysis showed that the patient, his mother, and brother 1 carried a CFH-p.R1215Q mutation, but his mother and brother 1 have never had the episodes of aHUS.

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2K1 might have a genetic abnormality involved in the complement regulation system, inherited from her father.

3. Mild hemolysis: 25–50%. Six patients (3M1, 2J2, 3B1, 3C1, 2W1, and 3E1) were in this category. Three patients had predisposing mutations. Patients 3M1 and 2J2 had a p.I1157T mutation in the TED of C3 (described below) and Patient 3C1 had a p.D486Y mutation in the serine-threonine-rich region of THBD. Furthermore, two other patients (3B1 and 2W1) had potentially predisposing mutations: p.A311V in the transmembrane region of MCP, and p. N331D in the von Willebrand type A domain of CFB, respectively. Only one patient (3E1) had no abnormalities in the six candidate genes and the CFHR1 and CFHR3 proteins based on Western blot.

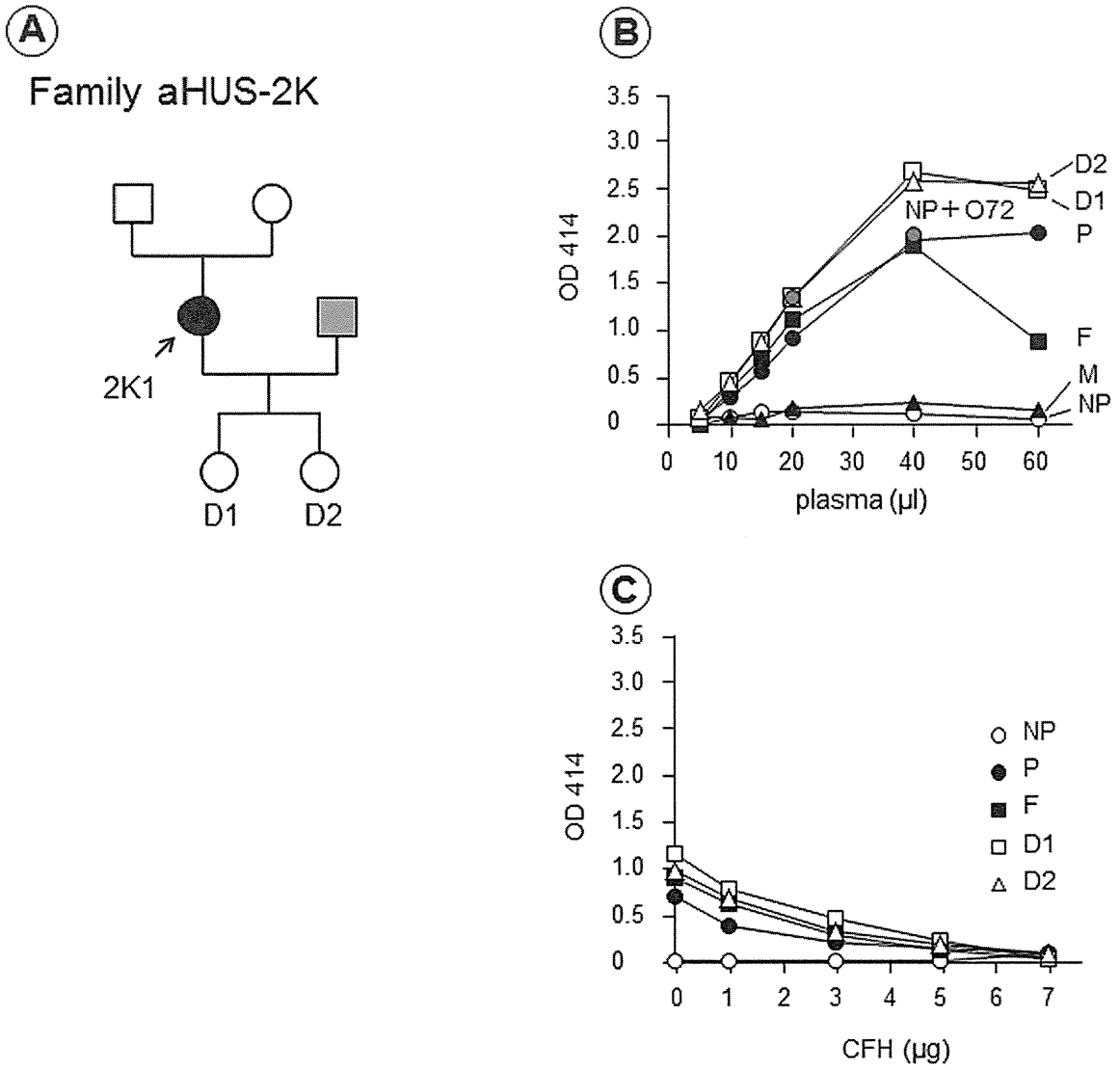

Fig 6. Family 2K with no predisposing or potentially predisposing mutations. (A) Patient 2K1 (female) developed atypical hemolytic uremic syndrome (aHUS) at the age of 35. Her husband (gray square) was not analyzed in this study. (B) The hemolytic assay showed that plasma from the patient (P), her father (F), and two daughters (D1 and D2) induced severe hemolysis, which was not observed in her mother (M). (C) The enhanced hemolysis detected in these four family members was corrected by the addition of purified complement factor H (CFH). However, no predisposing or potentially predisposing mutations were detected in Patient 2K1.

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4. No apparent hemolysis: <25%. There were 28 patients in this category, of whom 16 had C3 mutations. Surprisingly, among these 16 patients, 14 had the same predisposing mutation, p.I1157T in the TED of C3. The remaining two patients (2H1 and 2F1) had potentially predisposing mutations in C3, p.R425C and p.S562L, respectively. Two patients (2D1 and 2X1) had predisposing mutations in two transmembrane proteins. One had a p.Y189D mutation in the SCR3 domain of MCP, and the other had a p.D486Y mutation in THBD. Patient 3J1 had a potentially predisposing mutation, p.V231I in THBD. Curiously, Patient 3G1 having anti-CFH autoantibody, but without deletion in CFHR1 and CFHR3 proteins, was also categorized in this group. This finding was quite different from other patients having anti-CFH autoantibodies. Further, the antibody titer in 3G1 (5,995 AU/mL) determined by quantitative ELISA was higher than that of 2Y1 (360 AU/mL) and 2G1 (4,813 AU/mL) (S3 Table). Thus, the

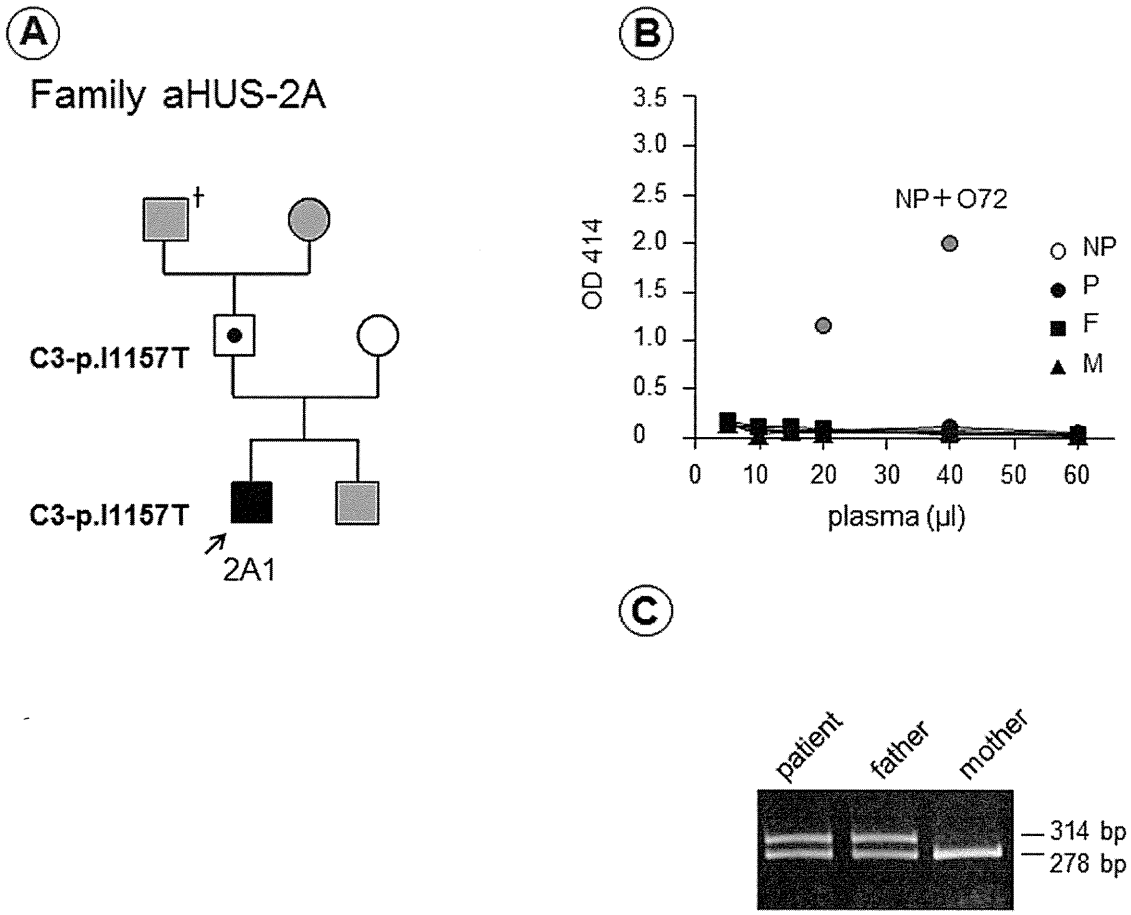

Fig 7. Family 2A with a C3-p.I1157T mutation. (A) Patient 2A1 (male) had six bouts of atypical hemolytic uremic syndrome (aHUS), at the age of 9, 15, 18, 22 and 29 (twice). Gray squares or circle indicate individuals who were not analyzed in this study. (B) The hemolytic assay showed that neither patient (P) nor his parents (father: F and mother: M) had appreciable hemolysis. However, Patient 2A1 and his father carried the same predisposing mutation p.I1157T in C3, confirmed by restriction fragment length polymorphism (RFLP) analysis (C) and direct DNA sequencing.

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discrepancy between the degrees of hemolysis and antibody titer might be attributable to the epitope specificity of anti-CFH autoantibodies. On the other hand, Patient 3G1 had low C3 level (30 mg/mL) (S1 Table), which might contribute to a low degree of hemolysis.

As a consequence, the most frequent genetic abnormality associated with congenital aHUS in Japan, p.I1157T in C3, was unable to be detected by the hemolytic assay. In Fig 7A, we showed the family of Patient 2A carrying a heterozygous C3-p.I1157T mutation as a representative example. Neither the patient nor his family members had enhanced hemolysis (Fig 7B), but the RFLP analysis of C3-p.I1157T clearly indicated that the patient and his asymptomatic father carried the same heterozygous predisposing mutation (Fig 7C), which was also confirmed by direct DNA sequencing. The results of the RFLP analysis in all 45 patients are shown alongside in the hemolytic assay results (Fig 3). These results clearly indicated whether the patient had a heterozygous or homozygous mutation in C3-p.I1157T or not, and 16 carriers were easily diagnosed by the RFLP analysis. Thus, in total a high frequency of a single C3 gene mutation (36%, 16/45) was identified in our aHUS cohort.

Most interestingly, a geographical distribution of gene mutations of aHUS patients showed that the patients carrying the C3-p.I1157T mutation were only found in an extremely restricted

area (Kansai district including Mie [31], Nara, Kyoto, and Osaka) and not found in other areas of West Japan (S1 Fig).

Discussion

Rapid differential diagnosis of TMA is needed in clinical practice to select the optimal therapeutic approach. Evaluations of ADAMTS13 activity and STEC infection are often necessary, both of them usually can be done within a few hours. Further, comprehensive genetic analysis has been proposed as being able to consistently diagnose aHUS in approximately 70% of patients, but it usually takes at least several weeks, resulting in almost no clinical utility during emergencies. Since a relatively high proportion (approximately 20–30%) of mutations occurs in CFH among Caucasian aHUS patients [5, 11], the usefulness of a qualitative hemolytic assay using sheep RBCs has been postulated [12, 13]. However, it appears that the hemolytic assay has not been commonly used in many laboratories. This may be due to unstable viability of sheep RBCs over long-term storage at 4°C, unreal 100% hemolysis of sheep RBCs exposed to distilled water, and the type of blood specimens used, such as serum, EDTA plasma, or citrated plasma. Here, we have shown that serum is not a good specimen for the hemolytic assay, and EDTA plasma cannot be used in the ADAMTS13 activity assay.

In this study, we have successfully generated a novel murine anti-CFH mAb O72, which induces severe hemolysis at 200 μg/ml (final concentration) when spiked into an assay mixture of normal human plasma and sheep RBCs. The mAb O72 epitope resides in SCR18, which could explain its ability to block CFH binding to the sialic acid-rich cell surface of sheep RBCs. Interestingly, the degree of hemolysis induced by normal plasma spiked with mAb O72 is almost the same as that mediated by plasma from a patient with a heterozygous CFH-p.R1215Q mutation. In both these plasma samples, enhanced hemolysis caused by the presence of non-functional CFH was corrected by the addition of purified CFH in a dose-dependent manner, and totally eliminated at high concentrations. By using the novel quantitative hemolytic assay, we have retrospectively analyzed 45 aHUS patients in our cohort. The aHUS is an extremely rare disease, and so far only one hundred patients have been diagnosed across Japan. We are expecting to find merely 10–15 new patients every year, and therefore the prospective analysis is difficult to perform at the moment. This is why we performed retrospective analysis in this study.

Based on the quantitative hemolytic assay, 11 of 45 aHUS patients in Japan had more than 50% (moderate-to-severe) hemolysis, in whom the following gene mutations or abnormalities were identified: two patients (2I1 and X1) with CFH-p.R1215Q, one patient (2M1) with CFH-p.R1215G, five patients (3T1, W1, 2Y1, 2G1 and 2P1) with anti-CFH Ab, one patient (3O1) with C3-p.K1105Q, and two patients (3A1 and 2K1) without any gene mutations. Severe hemolysis was also identified in three asymptomatic family members (Patient 2I's mother and brother 1, and Patient X1's father) with a heterozygous CFH-p.R1215Q mutation. Presently, we are unable to address whether they are asymptomatic carriers or latent patients.

An interesting family (aHUS-2K) was identified by the hemolytic assay as shown in Fig 6. In this family, plasmas of the patient, her father, and two daughters caused severe hemolysis, while the mother's plasma did not cause hemolysis in this assay. However, predisposing mutations were not identified in Patient 2K1, and anti-CFH autoantibodies were negative in all members of this family tested. Roumenina et al. [13] reported that the presence of a hybrid CFH/CFHR1 gene caused appreciable hemolysis of sheep RBCs. Thus, this patient might have a hybrid CFH/CFHR1 gene, or hitherto unrecognized genetic defect, inherited from the paternal side. Notably, without this novel hemolytic assay, evidence of complement-associated

Table 1. Comparison of genetic or acquired abnormalities among Western countries and Japan.

	Frequency			
Genetic or acquired abnormalities	**Noris et al (n = 273) Italy**	**Maga et al (n = 144) USA**	**Fremeaux-Bacchi et al (n = 214) France**	**Yoshida et al (n = 45) Japan**
CFH	24%	27%	28%	7%
anti-CFH Ab	3%	ND	7%	13%
C3	4%	2%	8%	42%
MCP	7%	5%	9%	5%
THBD	5%	3%	0%	7%
CFB	0.4% (n = 1)	4%	2%	2%
CFI	4%	8%	8%	0%
unidentified	~30%	54%	34%	24%

The frequency of each genetic or acquired abnormality in our aHUS cohort in Japan was compared to that in three other cohorts (Italy [32], USA [33], France [34]). The data reported by Noris et al. included 47 secondary aHUS patients. Sixty-percent of aHUS patients in our cohort were enrolled from West Japan. In the data of Japan, both predisposing and potentially predisposing mutations were counted. Although two patients (2V1 and H2) had two predisposing mutations (C3-p.I1157T and THBD-p.D486Y), they were counted as C3 group.

CFH: complement factor H, Ab: antibody, C3: complement component C3, MCP: membrane cofactor protein, THBD: thrombomodulin, CFB: complement factor B, CFI: complement factor I, ND: no data

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aHUS in this patient would have been difficult to obtain. The C3-p.K1105Q mutation in Patient 3O1 is a unique exception, which will be discussed later.

The remaining 34 aHUS patients had less than 50% (almost none-to-mild) hemolytic reactions, in whom the following predisposing gene mutations were identified: 16 patients with C3-p.I1157T, two patients (3C1 and 2X1) with THBD-p.D.486Y, one patient (2D1) with MCP-p.Y189D and nine patients with no mutations and no CFH autoantibodies. Moreover, one patient (3G1) had anti-CFH autoantibodies.

This is the first report that a patient with the C3 mutation, C3-p.K1105Q shows severe hemolytic activity (Figs 3 and 4), because usefulness of the hemolytic assay has been mostly referred to functional defects of CFH in previous publications [12, 13]. The C3-p.K1105Q mutation is located in the TED of C3 as shown in S2A Fig. A structural model of the complex of C3d with CFH-SCR19-20 showed that the C3-p.K1105Q mutation was positioned at the contact interface between C3d and CFH-SCR19, suggesting the defect of C3d binding to CFH-SCR19 (S2C Fig). In contrast, the p.I1157T mutation identified in 16 aHUS patients with low (less than 50%) hemolytic activity was positioned at the contact interface between C3b and CFH-SCR4, suggesting the defect of C3b binding to CFH-SCR1-4 (S2B Fig). Based on these differences, we assume that C3-p.K1105Q mutant with defective binding to CFH-SCR19 could induce the severe hemolysis.

We also identified interesting patients of 3B1 with MCP mutation and 3C1 with THBD mutation with mild hemolysis (25–50%), because both of these transmembrane proteins do not directly affect *in vitro* hemolysis. Thus, presently the mechanism of mild hemolysis shown in these plasma samples left unaddressed.

We found that the frequency of C3 mutation in aHUS patients in Japan (42%, 19/45) was much higher than that in other aHUS cohorts in Western countries [32–34] (Table 1). Moreover, Schramm et al. recently reported the frequency of C3 mutation in the French, Italian, UK,

and USA aHUS cohorts to be 11.4%, 4.6%, 6.1% and 4.5%, respectively [35]. As shown in S1 Fig, the patients with C3-p.I1157T mutation were only found in the Kansai district, West Japan [31] and not found in other areas of Japan, suggesting a reflection of 'founder effect'. Now, we have started to perform a cohort study of aHUS in East Japan.

In summary, we report that 24% (11/45) of aHUS patients in Japan have moderate-to-severe (≥50%) hemolysis, whereas the remaining 76% (34/45) patients had mild or almost no hemolysis (<50%). In the former group, this was largely attributed to CFH abnormalities, whereas in the latter group, 16 patients carried C3-p.I1157T mutation, which was identified by a simple RFLP analysis. We assume that this novel quantitative hemolytic assay would be more useful for the diagnosis of aHUS in Caucasians, who have a higher proportion of CFH mutations.

Supporting Information

S1 Fig. Geographical distribution of patients with atypical hemolytic uremic syndrome (aHUS) in Japan. Each aHUS patient is described as a black or red circle. Sixteen patients with C3-p.I1157T mutation shown by red circles were found only in the Kansai district, including Mie, Nara, Kyoto, and Osaka prefectures. Interestingly, patients carrying C3-p.I1157T mutation were not found in other areas of West Japan, which may indicate a reflection of 'founder effect'.
(TIF)

S2 Fig. Locations of the C3-p.I1157T and C3-p.K1105Q mutations in the C3b-complement factor H (CFH)-SCR1-4 complex and the C3d-CFH-SCR19-20 complex. (A) A structural model of the complex of C3b and short consensus repeat (SCR) 1–4 of CFH (ID: 2WII). The α and β chains of C3b are shown in cyan and blue, respectively and the SCR1-4 domains are depicted with gray, red, orange, and magenta, respectively. The I1157 and K1105 residues shown by the grey-labeled side chains are located in the thioester-containing domain (TED) in C3b. **(B) Close-up view of the region around the contact interface between C3b and SCR1-4.** The p.I1157T mutation identified in 16 patients with low (less than 50%) hemolytic activity, but not the p.K1105Q mutation identified in a patient with high (100%) hemolytic activity, is positioned at the interface between C3b and SCR1-4 and would interfere the C3b binding to CFH-SCR1-4. **(C) A structural model of the complex of C3d and SCR19-20 of CFH (ID: 3OXU).** C3d, SCR19, and SCR20 are depicted with cyan, blue, and yellow, respectively. The p.K1105Q mutation, but not the p.I1157T mutation, is positioned at the interface between C3d and SCR19-20, and would interfere the C3b binding to CFH-SCR19-20. Diagram was generated with the PyMOL molecular visualization system.
(TIF)

S1 Table. The levels of C3 and complement factor H (CFH) protein in 43 of 45 patients with atypical hemolytic uremic syndrome (aHUS). C3 level was determined by immune-nephelometry (SRL, Inc., Japan), and CFH level was measured by Laurell's immunoelectrophoresis using rabbit anti-CFH serum prepared in our laboratory. The levels of C3 and CFH in 43 aHUS patients and the mean ± standard deviation of these patients and normal plasma from 20 healthy individuals were described. CFH: complement factor H, C3: complement component C3, NP: normal plasma, ND: not determined
(TIF)

S2 Table. Plasmids and primers for expression of recombinant complement factor H (CFH) and its domains. SCR: short consensus repeat, CFH: complement factor H, Pk tag: GKPIPNPLLGLDST sequence, aa: amino acids.
(TIF)

S3 Table. Characteristics of five of six patients with anti-complement factor H (CFH) autoantibodies according to ELISA. Determination of CFH autoantibody titer was performed by CFH-IgG ELISA kit (Abnova). Antibody titer was calculated according to the manufacturer's protocol by using standard curve. AU: arbitrary unit, ND: not determined.
(TIF)

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Author Contributions

Conceived and designed the experiments: TM MM TK YF. Performed the experiments: YY HSI YU YO. Analyzed the data: YY TM MM HSI YU YO TK YF. Contributed reagents/materials/analysis tools: TM MM TK YF. Wrote the paper: YY TM MM YO TK YF.

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Poor responder to plasma exchange therapy in acquired thrombotic thrombocytopenic purpura is associated with ADAMTS13 inhibitor boosting: visualization of an ADAMTS13 inhibitor complex and its proteolytic clearance from plasma

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BACKGROUND: Plasma exchange (PE) is the first-line treatment for primary acquired thrombotic thrombocytopenic purpura (aTTP) with severe deficiency of ADAMTS13 activity (ADAMTS13:AC). Some patients are poor responders to PE, raising concern over multiple pathogenetic pathways.

STUDY DESIGN AND METHODS: Based on 52 aTTP patients in our national cohort study, we monitored plasma levels of ADAMTS13, clinical and laboratory findings, and outcomes. In a representative poor responder to PE, we examined an ADAMTS13 inhibitor (ADAMTS13:INH) complex in plasma milieu, by means of a large-pore isoelectric focusing (IEF) analysis.

RESULTS: Of 52 aTTP patients, 20 were good responders and 32 were poor responders. In the latter group, plasma ADAMTS13:AC levels never increased to more than 10% of normal during 14 days after PE initiation. Mean (±SD) plasma ADAMTS13:INH titers (Bethesda unit/mL) were 5.7 (±4.5) before PE, but decreased to 1.4 (±0.8) on the fourth PE day and then remarkably increased to 14.8 (±10.0) on the 10th PE day, termed "inhibitor boosting," and then slowly decreased to undetectable level over 1 month. On admission, none of the routinely available clinical and laboratory markers differentiated these two groups. However, elevated pre-PE levels of ADAMTS13:INH were correlated with a poor response. We visualized an ADAMTS13:INH (immunoglobulin G) complex in a patient plasma by an IEF analysis and found proteolytic fragment of ADAMTS13 antigen by a two-dimensional IEF and sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis.

CONCLUSION: Findings from this cohort of aTTP patients demonstrated that inhibitor boosting often occurs in aTTP patients in Japan. Poor responders could be predicted by elevated pre-PE ADAMTS13:INH levels on admission, but not by routinely collected clinical or laboratory data.

Thrombotic thrombocytopenic purpura (TTP), a life-threatening generalized disorder originally characterized by a pentad of thrombocytopenia, microangiopathic hemolytic anemia, renal dysfunction, neurologic signs, and fever,[1] is now primarily defined by severe deficiency of von Willebrand factor (VWF)-cleaving protease, termed ADAMTS13 (a

ABBREVIATIONS: ADAMTS13:AC = ADAMTS13 activity; ADAMTS13:AG = ADAMTS13:antigen; ADAMTS13:INH = ADAMTS13 inhibitor; aTTP = primary acquired thrombotic thrombocytopenic purpura; IEF = isoelectric focusing; PE = plasma exchange; UL = unusually large; VWFM = von Willebrand factor multimer.

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disintegrin-like and metalloprotease with thrombospondin Type 1 motifs 13) accompanied by thrombocytopenia.[2-4] The ADAMTS13 specifically cleaves unusually large VWF multimer (UL-VWFM) with hyperaggregability of platelets (PLTs)[5] and down regulates VWF function. Deficiency of ADAMTS13 activity (ADAMTS13:AC) is caused by either gene mutations in or acquired autoantibodies to this enzyme,[6] occasionally without a known cause, termed primary acquired TTP (aTTP).[7]

Plasma exchange (PE) has long been the first-line treatment for aTTP, often with an adjunct of corticosteroid and corticosteroid pulse therapy,[8] PE removes ADAMTS13 inhibitor (ADAMTS13:INH), UL-VWFM, and inflammatory cytokines that mediate UL-VWFM release from vascular endothelial cells and replenishes ADAMTS13 and regular-sized VWFM required for normal hemostasis. Patients with aTTP frequently take several days to weeks of PE before PLT counts recover. However, one population of aTTP patients often shows an initial increase in the PLT count that is paradoxically followed by secondary thrombocytopenia. These patients have been categorized as having "PE-refractory aTTP," but the pathogenesis of secondary thrombocytopenia is unclear. Böhm and colleagues[9] reported an increase in ADAMTS13:INH titers after PE, but no systematic studies on this potentially important issue have been conducted. In addition, 30% to 50% of aTTP patients will subsequently relapse, although these relapses occur at a time point that is much longer than the secondary thrombocytopenia described herein.[6,10] Due to concerns over both short-term thrombocytopenic events and long-term aTTP relapse, the anti-CD20 rituximab, which removes inhibitory immunoglobulin (Ig)G-producing B lymphocytes from circulation,[11,12] has been used recently to treat aTTP patients in conjunction with PE and corticosteroid, in an effort to reduce IgG of ADAMTS13:INH with the goal of improving short-term responses as well as decreasing the rate of relapse.

We have recently shown that ADAMTS13 antigen (ADAMTS13:AG) in the plasma milieu consisted of three groups of bands with different isoelectric points (pI); Band I (pI 4.9 to 5.6) represents ADAMTS13 not bound to VWF, Band II (pI 5.8 to 6.7) remains unaddressed, and Band III (pI 7.0 or 7.5) corresponds to ADAMTS13 bound to larger VWFM.[13] Further, during 1998 to 2012 we identified 52 patients with new onset of aTTP, whose plasma samples were followed for serial ADAMTS13 level measurements more than three times within 2 weeks after initiation of PE. These patients were classified into two groups based on the pre-PE levels of plasma ADAMTS13:AC measured on the 14th day after PE initiation; 20 good ADAMTS13:AC responders (ADAMTS13:AC $\geq$10%) and 32 poor ADAMTS13:AC responders (ADAMTS13:AC $<$ 10%). We found that poor ADAMTS13:AC responders were uniformly associated with a tremendous increase of ADAMTS13:INH titers (IgG1 subclass) on the 10th PE day, termed "inhibitor boosting." Further, using an isoelectric focusing (IEF) gel we visualized for the first time a complex of ADAMTS13:AG and ADAMTS13:INH (IgG), that underwent proteolytic cleavage followed by clearance from circulation. These studies may help to understand how treatment shortly after PE with corticosteroid and rituximab infusion works in aTTP patients who otherwise may develop secondary thrombocytopenia due to inhibitor boosting.

MATERIALS AND METHODS

Fifty-two aTTP patients enrolled in this national cohort study

Since 1998, our laboratory at Nara Medical University has served as a nationwide referral center for thrombotic microangiopathy that analyzes ADAMTS13 as requested by clients across Japan. Using a chromogenic assay for ADAMTS13:AC,[14] between January 2004 and March 2012, we were able to identify 184 new patients (89 males and 95 females) with aTTP, based on the following criteria: severe deficiency of plasma ADAMTS13:AC (<10% of normal) with positive ADAMTS13:INH (>1.0 Bethesda unit [BU]/mL), but without the underlying diseases. Of 184 aTTP patients, 52 were able to have follow-up determination of plasma ADAMTS13:AC levels more than three times at 3- to 5-day intervals within 14 days after PE initiation and were enrolled in this study (see Tables S1and S2, available as supporting information in the online version of this paper).

Patient outcome definitions

The study definitions were the same as those reported by Kremer Hovinga and colleagues[15] and Froissart and colleagues.[16] Remission was defined as a complete resolution of laboratory and clinical data after cessation of PE. Durable remission was defined as laboratory and clinical resolution of aTTP evaluated at least 30 days after the first day of PLT count recovery (>150 $\times$ 10^9/L; this period included the time on maintenance PE). Relapse was considered a new episode of TTP if a patient had achieved a durable remission and subsequently experienced a laboratory relapse. Death from TTP was defined as any death that occurred within 30 days after a diagnosis of TTP.

Assays for plasma levels of ADAMTS13:AC, ADAMTS13:INH, and ADAMTS13:AG

Plasma levels of ADAMTS13:AC were measured by chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA)[14] with a detection limit of 0.5% of normal, and the ADMTS13:INH titers were determined by means of the Bethesda method[17] using heat-inactivated patient plasmas, as previously described. One BU is defined as the amount necessary to reduce ADAMTS13:AC to 50% of control levels, and the value of at least 1.0 BU/mL was

assumed to be positive. Further, plasma levels of ADAMTS13:AG were measured using sandwich ELISA.[18]

Titers of anti-ADAMTS13 IgG, IgM, and IgA autoantibodies and IgG1-4 subclasses

Assays were performed as previously described,[19] with the following modifications. First, microtiter plates (Nunc Immuno Maxisorp, Nunc A/S, Roskilde, Denmark) were coated with 100 μL of human recombinant ADAMTS13 (2 μg/mL) dissolved in a coating buffer (carbonate-bicarbonate buffer, pH 9.6, Sigma C-3041, Sigma-Aldrich, St Louis, MO]) and left overnight at 4°C. On the next day, 250 μL of blocking buffer (Pierce protein-free T20, Thermo Fisher Scientific, Inc., Rockford, IL) was added to the plates and incubated for 2 hours at room temperature. Then, 100 μL of each diluted test sample was added to each well and left for 3 hours at room temperature. After incubation, 100 μL of diluted horseradish peroxidase–conjugated goat anti-human IgG, IgG1-4, IgM, or IgA was added, and the plates were incubated for 2 hours at room temperature. Finally, 100 μL of 3,3′,5,5′-tetramethylbenzidine-H_2O_2 solution (3,3′,5,5′-tetramethylbenzidine substrate kit, Thermo Fisher Scientific Inc.) was added, and the absorbance was measured at 450 nm with a reference filter of 620 nm on a microplate reader (Thermo Fisher Scientific, Inc.). Between each step, the plates were washed with phosphate-buffered saline (PBS, pH 7.2) four times. Titers were calculated as a ratio of the sample optical density (OD) at each dilution to the normal human plasma OD at each dilution. Ratios were determined for each individual dilution and compared to the corresponding cutoff values, and the results are expressed as the titer. The titer of a sample corresponded to the last dilution at which the ratio was above the cutoff level.[19]

IEF using an agarose-acrylamide composite gel

In some experiments, ADAMTS13 in patient plasma was analyzed by IEF using a large-pore agarose-acrylamide composite gel as recently described in detail.[13] After IEF, the isolated proteins were electrophoretically transferred to nitrocellulose membranes, and then the blotted proteins were immunoreacted with anti-ADAMTS13 monoclonal antibody (WH2-11-1, epitope residing on the fourth thrombospondin Type 1 domain of ADAMTS13)[20] and visualized using a chemiluminescent detection kit (Perkin-Elmer Life Science, Boston, MA). With this method, ADAMTS13:AG from normal plasma consistently shows the following three groups of bands: a major Band I representing unbound ADAMTS13 with a pI of 4.9-5.6, as in the case of purified plasma-derived ADAMTS13, as well as Band II at pI 5.8-6.7 and Band III at pI 7.0/7.5. The composition of Band II is not well defined yet, but Band III was shown to be a complex of ADAMTS13:AG and high-molecular-weight VWFM.[13]

Therapeutic regimen

The basic therapeutic regimen for our cohort patients with aTTP consisted of PE, corticosteroids, and immunosuppressants such as rituximab (see Tables S1 and S2). All patients were initially treated with PE with fresh-frozen plasma at a dose of 40 to 60 mL/kg body weight once per day and mostly for the first 3 to 5 consecutive days. However, the subsequent regimen of PE therapy was variable based primarily on laboratory findings of patients (PLT count and serum lactate dehydrogenase [LDH]) and presence of neurological findings.

PE therapy was usually accompanied with corticosteroid therapy. In our cohort, 30 patients received high-dose methylprednisolone (0.5-1 g/day) pulse therapy, of whom 23 patients received it commonly for 3 consecutive days for 1 to 3 days after PE and seven patients received it beyond this period. An additional 13 patients who did not receive corticosteroid pulse therapy received per os administration of predonisolone (0.5-1 mg/kg/day). One patient did not have corticosteroid therapy. For the remaining eight patients, we were unable to retrieve information on corticosteroid therapy from the physicians-in-charge.

For immune suppressants, rituximab infusion was most frequently used (18/52 patients with aTTP). The rituximab dose was 375 mg/m^2 weekly for 4 weeks, although one patient (Patient 34) died soon after the first rituximab infusion and one patient (Patient 26) received five infusions of rituximab. Patient 28 (female) was an exception, who received a second course of rituximab therapy for 75 to 96 days (a total of eight rituximab infusions), as the patient had persistence of high titers of ADAMTS13:INH (peak of 42.6 BU/mL). An additional 10 patients received vincristine (1-2 mg/body per day) one to four doses administered 1 week apart, and four patients received one or two cycles of cyclophosphamide pulse therapy (500 mg/body/day). Therapies administered to three additional patients included intravenous immunoglobulin (one patient), one dose of cyclosporine (one patient), and aspirin (one patient).

Statistical analysis

Laboratory data are expressed as the mean ± SD. Comparisons between good ADAMTS13:AC responders and poor ADAMTS13:AC responders were analyzed using the Mann-Whitney U test or chi-square test. All analyses were carried out using with computer software (StatView, SAS Institute, Inc., Cary, NC). A p value of less than 0.05 was considered significant.

RESULTS

Two patient groups of aTTP: good ADAMTS13:AC responders and poor ADAMTS13:AC responders to PE

Follow-up clinical and laboratory findings were available for all 52 aTTP patients (between 7 and 2607 days after

TABLE 1. Comparison of laboratory markers: good ADAMTS13:AC responders and poor ADAMTS13:AC responders to PE therapy

	ADAMTS13:AC (%) on 14th day after PE		
Variable	≥10% (good responders)	< 10% (poor responders)	p value
Number	20	32	
Age on admission (years)	53 (1-81)	48 (13-84)	0.44
Sex			
Female	13 (65%)	19 (59%)	0.91
Male	7 (35%)	13 (41%)	
PE (times) total	7.5 (3-15)	14.3 (4-31)	<0.01
For 14 days	6.8 (3-11)	9.9 (4-14)	<0.01
After 14 days	0.7 (0-6)	4.5 (0-20)	<0.01
Rituximab therapy	3	15	<0.05
Steroid and steroid pulse therapy	18	30	0.62

the first admission). These follow-up data are summarized in Tables S1 and S2. The 52 aTTP patients were classified into two groups based on comparison of plasma levels of ADAMTS13:AC measured 14 days after initiation of PE: 20 aTTP patients are characterized as good ADAMTS13:AC responders (ADAMTS13:AC ≥10%) and 32 aTTP patients are characterized as poor ADAMTS13:AC responders (ADAMTS13:AC < 10%). Characteristics of these two aTTP patient groups are shown in Table 1. Most of the two patient groups received corticosteroids. Eighteen aTTP patients received rituximab, which was administered to poor ADAMTS13:AC responders more frequently than good ADAMTS13:AC responders ($p < 0.05$).

In good ADAMTS13:AC responders (Fig. 1, left), plasma levels of ADAMTS13:AC (mean ± SD) increased to 15.0 ± 10.9% on the third day of PE, with a slight dip on the 10th day of PE, and then did not decrease below 10% within 14 days after PE initiation. As for plasma ADAMTS13:INH titer in good ADAMTS13:AC responders, mean ± SD ADAMTS13:INH levels were 2.5 ± 1.5 BU/mL before PE, decreasing to 0.8 ± 0.4 BU/mL on the fourth PE day, and then increasing to almost pre-PE levels (2.7 ± 4.4 BU/mL) on the 10th PE day, followed by a consistent decrease to undetectable ADAMTS13:INH levels (<0.5 BU/mL) on the 14th PE day. PLT recovery began 1 to 2 days later and mean PLT count increased to 180 × 10^9/L on the 6th PE day, followed by a slight dip, and the mean PLT counts increased again to more than 150 × 10^9/L on the 14th PE day.

In poor ADAMTS13:AC responders (Fig. 1, right), plasma levels of ADAMTS13:AC never increased to more than 10% of normal as measured at the 14th day after PE initiation. Further, mean ± SD plasma ADAMTS13:INH titers were 5.9 ± 4.5 BU/mL before PE initiation, but decreased to 1.4 ± 0.7 BU/mL on the 4th PE day, and then increased to 14.8 ± 0.7 BU/mL on the 10th PE day. This is termed inhibitor boosting. Mean ADAMTS13:INH titers subsequently decreased to undetectable levels (<0.5 BU/mL) over a 4-week period after PE initiation. Among these aTTP patients, recovery of PLT counts was slow, and the PLT count was not more than 150 × 10^9/L in any of these patients by the 22nd PE day.

Sequential analysis of ADAMTS13:INH immunoglobulin class and IgG subclasses

A previous report[19] indicated clinical significance of the IgG4 subclass of ADAMTS13:INH (IgG) rather than others, and therefore we here sequentially measured the titers of immunoglobulins and IgG subclasses in six aTTP patients, including two good ADAMTS13:AC responders (Patients 1 and 3) and four poor ADAMTS13:AC responders (Patients 26, 36, 39, and 40; Fig. 2).

Both good ADAMTS13:AC responder patients had IgG antibodies (IgG1 in both patients, IgG2 and IgG4 classes in one patient) and one had IgA antibodies. Over time, IgG and IgA antibody titers correlated with ADAMTS13:INH titers. Further, all four patients in poor ADAMTS13:AC responders had IgG antibodies (IgG1 in four patients, IgG2 in two patients, and IgG4 in two patients) and one also had IgA antibodies, but none had IgM antibodies. Over time, IgG and IgA antibody titers correlated with ADAMTS13:INH titers. Thus, in both patient groups the ADAMTS13:INH titers were consistently correlated with the IgG1 titers, and inconsistently with the titers of other IgG subclasses.

Visualization of ADAMTS13:AG and its complex with ADAMTS13:INH (IgG) in plasma milieu during treatment

Plasma ADAMTS13:INH titers in aTTP patients correlated with titers of the IgG1 subclass. Further, despite severe deficiency of ADAMTS13:AC (<0.5% of normal) in all of our aTTP patients, these patients generally had low but variable levels of plasma ADAMTS13:AG (<0.1% to 84.8% of normal; Tables S1 and S2).

Most of the aTTP patients on admission had IEF patterns that differed from those of normal individuals (Fig.

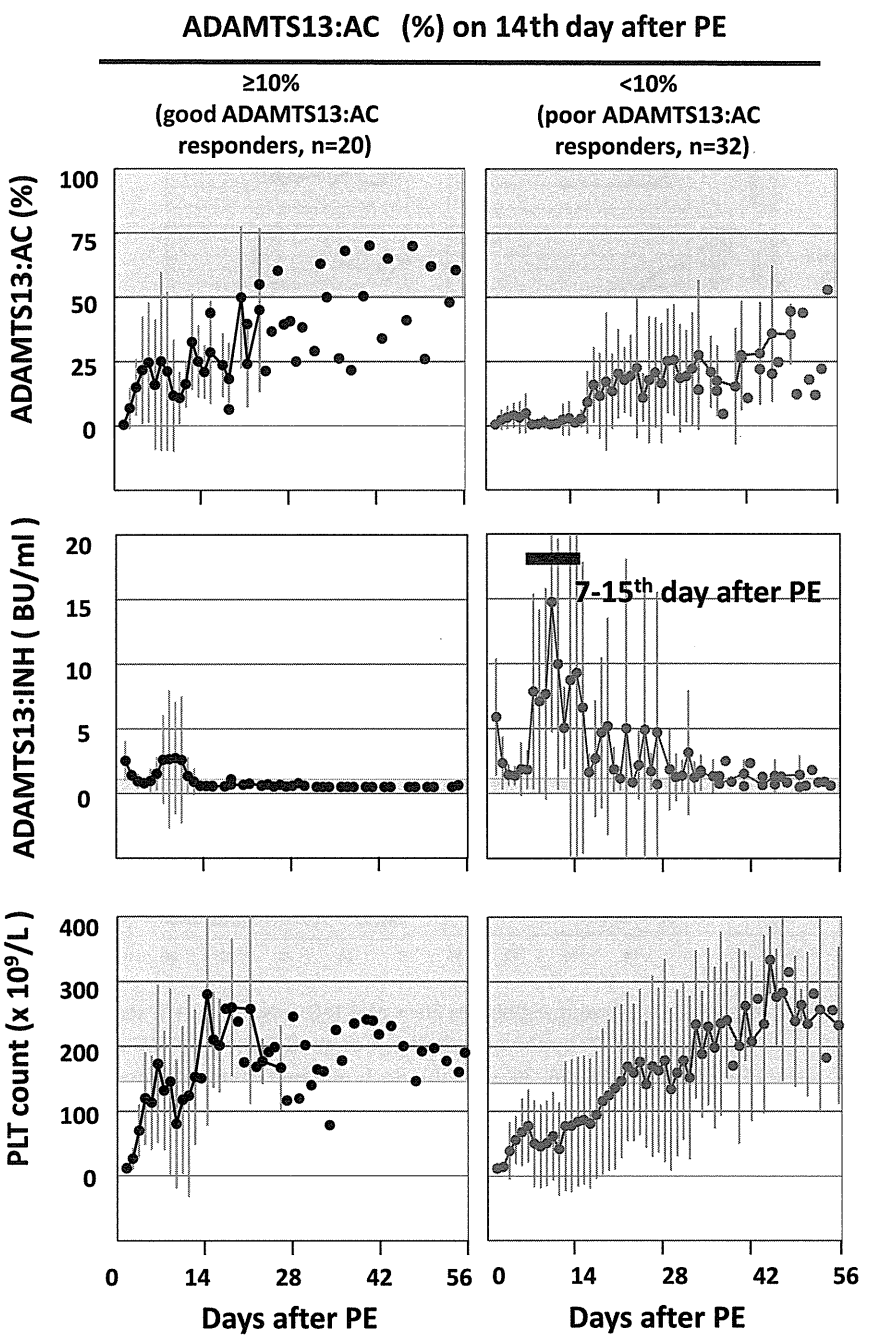

Fig. 1. Changes of plasma levels of ADAMTS13:AC and ADAMTS13:INH and PLT counts in 20 good ADAMTS13:AC responders (left) and 32 poor ADAMTS13:AC responders to PE (right). Shaded areas show normal ranges, and the vertical bars indicate values of mean ± SD. In good ADAMTS13:AC responders (left), plasma levels of ADAMTS13:AC increased to 15.0 ± 10.9% on the third PE day and then did not decrease below 10% within 14 days after PE initiation. Plasma ADAMTS13:INH titers decreased on the fourth PE day and then returned to almost pre-PE levels on 10th PE day, followed by a consistent decrease to the undetectable level on the 14th PE day. PLT counts increased to normal levels on the sixth PE day, followed by a slight dip, and then returned to normal on the 14th PE day. In poor ADAMTS13:AC responders (right), plasma levels of ADAMTS13:AC never increased to more than 10% for 14 days after PE initiation. Further, plasma ADAMTS13:INH titer decreased from 5.9 ± 4.5 BU/mL before PE initiation to 1.4 ± 0.7 BU/mL on the fourth PE day and then remarkably increased to 14.8 ± 0.7 BU/mL on the 10th PE day, termed inhibitor boosting, followed by a slow decrease to undetectable level more than 1 month after PE initiation. A recovery of PLT counts was very slow, and the count reached more than 150 × 10^9/L on the 22nd PE day.

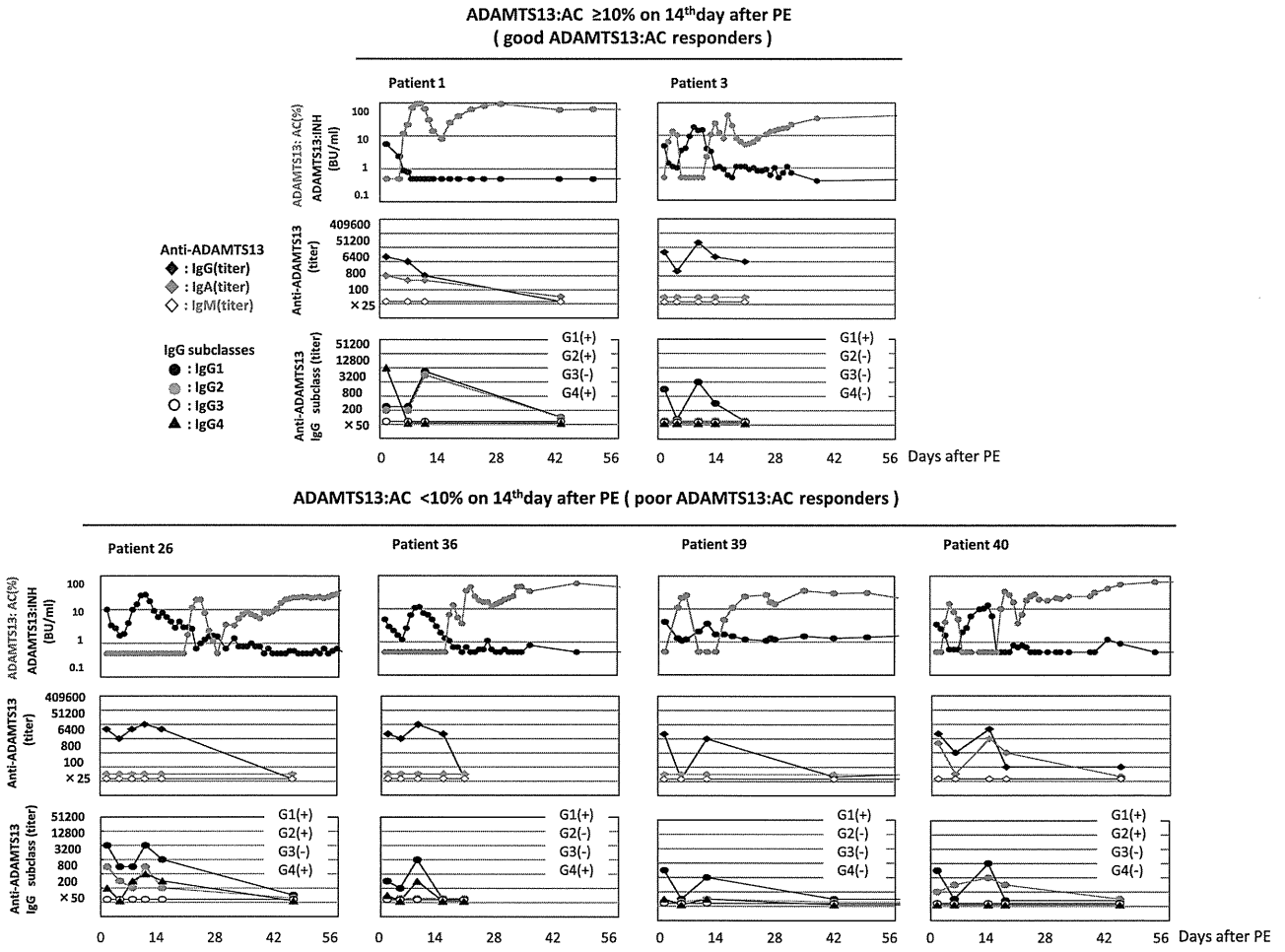

Fig. 2. Sequential analysis of ADAMTS13:INH immunoglobulin class and IgG subclasses. We sequentially analyzed the titers of immunoglobulins and IgG subclasses in six aTTP patients, including two good ADAMTS13:AC responders (Patients 1 and 3) and four poor ADAMTS13:AC responders (Patients 26, 36, 39, and 40). Both patients who were good ADAMTS13:AC responders had IgG antibodies (IgG1 in both patients, IgG2 and IgG4 classes in one patient) and one had IgA antibodies. All four patients who were poor ADAMTS13:AC responders had IgG antibodies (IgG1 in four patients, IgG2 in two patients, and IgG4 in two patients) and one had IgA antibodies. In both patient groups, the ADAMTS13:INH titers were consistently correlated with the IgG1 titers and inconsistently with the titers of other IgG subclasses.

S1, available as supporting information in the online version of this paper). Notably, among the 52 aTTP patients, three poor ADAMTS13:AC responders (Patients 22, 26, and 47) had undetectably low plasma level of ADAMTS13:AG (<0.1% of normal). We report laboratory information herein for Patient 26 (Fig. 3).

Before PE, Patient 26 had no visible band of ADAMTS13:AG on IEF gel, and 3 to 5 days after initiation of PE, a new ADAMTS13:AG band was detected at pI 6.0 to 7.3 (Band A). However, on Day 10, the ADAMTS13:AG band was not detected in accord with inhibitor boosting (Fig. 3B). During this period, plasma levels of ADAMTS13:AC were consistently below 0.5% of normal, suggesting that Band A was a complex of ADAMTS13:AG and ADAMTS13:INH (IgG). Band A was no longer detected after passing the patient's plasma through a protein G column to adsorb IgG (data not shown). After extensive treatment with PE, corticosteroid, and rituximab, ADAMTS13:AG patterns almost normalized on Day 100 after PE initiation, along with improvements of other aTTP-related clinical and laboratory findings. In fact, aTTP remission was achieved at this point. Furthermore, Band B with pI of more than 9.3 was identified as the degraded fragment of ADAMTS13:AG as shown by the presence of one ADAMTS13-related protein band (125 kDa) by two-dimensional analysis, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Fig. 3C).

Although remission was initially achieved in Patient 26, the patient subsequently experienced a relapse 2 years

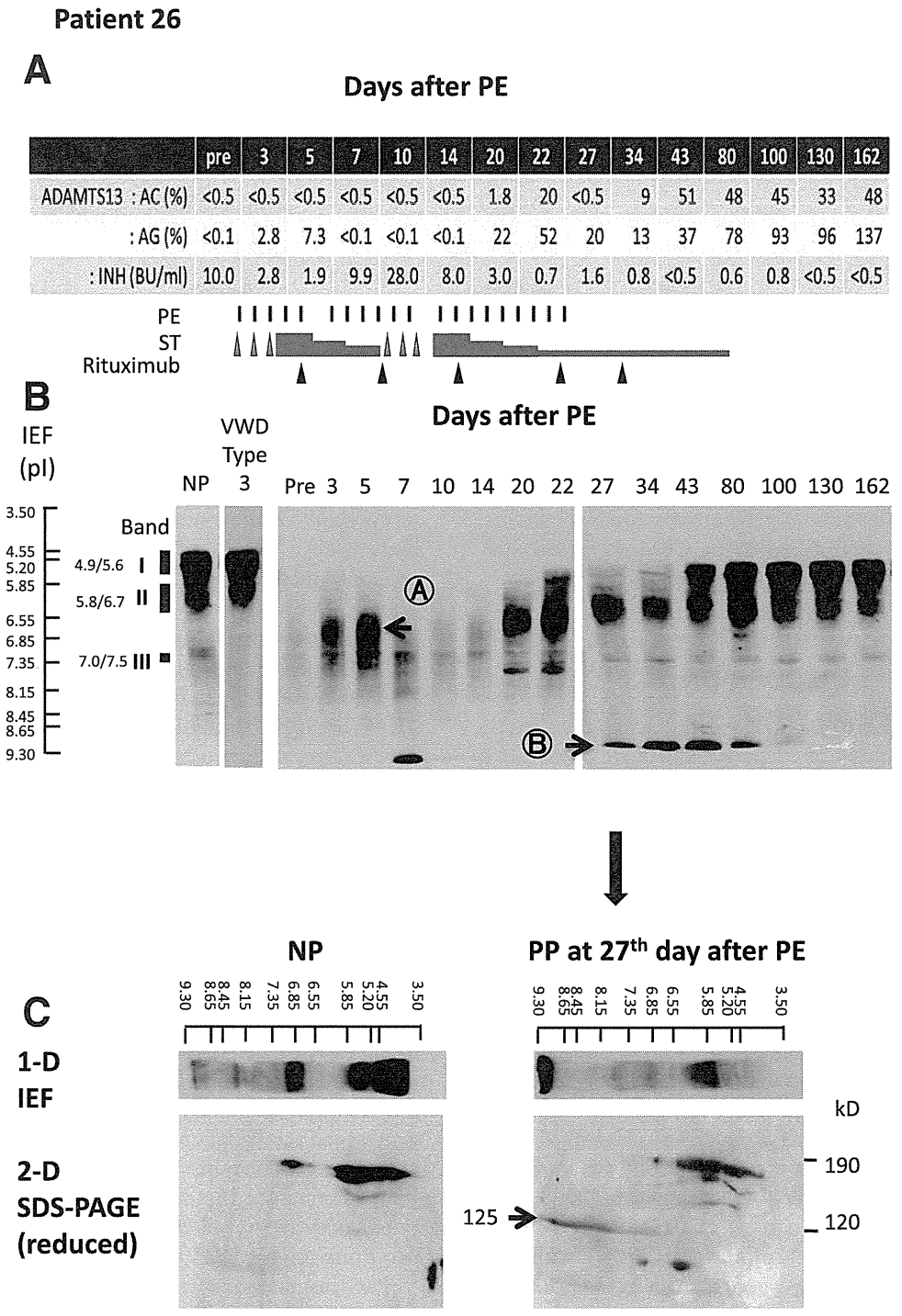

Fig. 3. Changes of ADAMTS13 variables and IEF analysis of plasma ADAMTS13:AG in patients with inhibitor boosting. Changes of ADAMTS13:AC, ADAMTS13:AG, and ADAMTS13:INH in addition to the therapy of patient with inhibitor boosting are shown in A. Before PE, this patient had no visible band of ADAMTS13:AG on IEF gel, and 3 to 5 days after initiation of PE, a new ADAMTS13 band at pI 6.0 to 7.3 (Band A) appeared, but on Day 10 it almost totally disappeared again in accord with inhibitor boosting as shown in B. After extensive treatment with PE, corticosteroid, and rituximab, ADAMTS13:AG patterns almost normalized on Day 100 after PE initiation. Band B with pI of more than 9.3 in B was identified as the degraded fragment of ADAMTS13:AG, shown by the presence of one ADAMTS13-related protein band (125 kDa) by two-dimensional analysis using SDS-PAGE under reducing conditions in C. VWD = von Willebrand disease; NP = normal plasma; PP = patient plasma.

TABLE 2. Comparison of laboratory markers between good ADAMTS13:AC responders and poor ADAMTS13:AC responders to PE therapy

	On admission			On 14th day after PE		
Marker	≥10% (good responders)	<10% (poor responders)	p value	≥10% (good responders)	<10% (poor responders)	p value
ADAMTS13						
:AC (%)	<0.5	<0.5		31.7 (12.0-68.0)	1.9 (<0.5-12.4)	<0.01
:AG (%)	12.3 (0.2-84.8)	7.9 (<0.1-37.9)	0.39	90.3 (36.0->100)	42.1 (0.1->100)	<0.01
:INH (BU/mL)	2.5 (1.0-5.6)	5.7 (1.1-20.0)	<0.01	0.54 (<0.5-0.9)	6.9 (0.7-51.0)	<0.01
Neurologic symptoms						
With	15 (15/20)	29 (29/32)		0 (0/20)	14 (14/32)	
Without	5 (5/20)	3 (3/32)		20 (20/20)	13 (13/32)	
PLTs ($\times 10^9$/L)	12.3 (3-36)	11.6 (3-54)	0.78	225.1 (70-772)	84.6 (3-330)	<0.01
LDH (IU/mL)	1696 (494-7768)	1262 (478-3126)	0.30	290 (165-891)	536 (161-1941)	<0.05
Hb (g/dL)	7.3 (4.2-14.3)	7.3 (4.9-10.1)	0.99	9.1 (7.0-11.9)	8.3 (5.1-12.6)	0.11
Blood urea nitrogen (mg/dL)	23.1 (9.5-58.7)	26.5 (8.8-122.0)	0.47	16.4 (3.7-32.0)	26.1 (9.3-83.0)	<0.05
Creatinine (mg/dL)	0.88 (0.31-1.73)	1.12 (0.39-7.70)	0.33	0.65 (0.20-0.97)	0.95 (0.43-4.30)	0.10

later, that was featured by thrombocytopenia and an ADAMTS:13 INH titer of 4.6 BU/mL. This relapse was successfully treated with PE and corticosteroids.

DISCUSSION

Using a large cohort of a TTP registry,[21] we conducted a retrospective analysis of 52 patients in Japan with aTTP. These patients were classified into two groups: 20 good ADAMTS13:AC responders and 32 poor ADAMTS13:AC responders, based on plasma levels of ADAMTS13:AC measured on the 14th day after PE initiation. aTTP patients who had ADAMTS13:AC levels less than 10% of normal on the 14th day after PE initiation were operationally considered to be "PE-refractory TTP." We also characterized the nature of ADAMTS13:INH antibodies and identified a complex of ADAMTS13:AG and ADAMTS13:INH (IgG), using a large-pore IEF gel analysis.[13] In interpreting our findings, several factors should be considered.

First, measured pre-PE levels of ADAMTS13:INH appeared to have prognostic implications for measured ADAMTS13:AC levels after PE. As shown in Table 2, on admission, none of the clinical and laboratory markers except for the pre-PE levels of ADAMTS13:INH could predict good ADAMTS13:AC responders versus poor ADAMTS13:AC responders.

Second, Ferrari and coworkers[19] reported that IgG4 was the most prevalent IgG subclass in their sample of patients with aTTP (90%, 52/58), followed by IgG1 (52%), IgG2 (50%), and IgG3 (33%). They further showed that patients with high IgG4 and undetectable IgG1 levels were more prone to relapse than those with low IgG4 levels and detectable IgG1; they proposed that IgG4 could be a potentially useful indicator of patients at risk of relapse. In our serial study of two good ADAMTS13:AC responders (without inhibitor boosting) and four poor ADAMTS13:AC responders (with inhibitor boosting), ADAMTS13:INH titers were most strongly correlated with the IgG class, especially the IgG1 subclass. The difference in IgG subclass findings between the study by Ferrari and colleagues[19,22] from Europe and our study from Japan is unclear, but predisposing genetic factors might be involved.

Third, we identified a complex of ADAMTS13:AG and ADAMTS13:INH (IgG) in patient plasma of poor ADAMTS13:AC responders, using a large-pore IEF gel analysis. This finding has not been reported previously, because most of aTTP patients have a low but significant plasma level of ADAMTS13:AG (as shown in Tables S1 and S2). We reported three patients who were poor ADAMTS13:AC responders (Patients 22, 26, and 47) who had low levels of plasma ADAMTS13:AG (<0.1% of normal) before PE. Using the patient post-PE plasma level for Patient 26, we reported that Band A was a complex of ADAMTS13:AG and ADAMTS13:INH (IgG), and Band B was the fragment of ADAMTS13:AG, as evidenced by a two-dimensional IEF and SDS-PAGE analysis (Fig. 3). These results indicate that the ADAMTS13:AG-ADAMTS13:INH complex undergoes proteolytic modification, by which ADAMTS13:AG disappears from circulation.

We conclude that ADAMTS13:INH boosting is a frequent occurrence noted among aTTP patients, primarily occurring among aTTP patients who were poor ADAMTS13:AC responders. Our results also identified that high ADAMTS13:INH titers (approx. > 5 BU/mL) measured before PE might be a good indicator to predict poor ADAMTS13:AC responders, for whom earlier administration of intensive immunosuppressive therapy such as rituximab appeared to facilitate reduction of the time and volume of PE. Further, durable remission of aTTP in patients who had measurable levels of ADAMTS13:AC may be more consistently identified by a new laboratory marker consisting of normal ADAMTS13:AG IEF patterns in the plasma milieu, but this does not guarantee freedom from relapse in the long term.

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CONFLICT OF INTEREST

FS and BP are employees of Baxter Bioscience. The other authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Isoelectric focusing (IEF) analysis of plasma ADAMTS13 antigen on admission in both patients of good ADAMTS13:AC responders and poor ADAMTS13:AC responders. ADAMTS13:AG exists in plasma