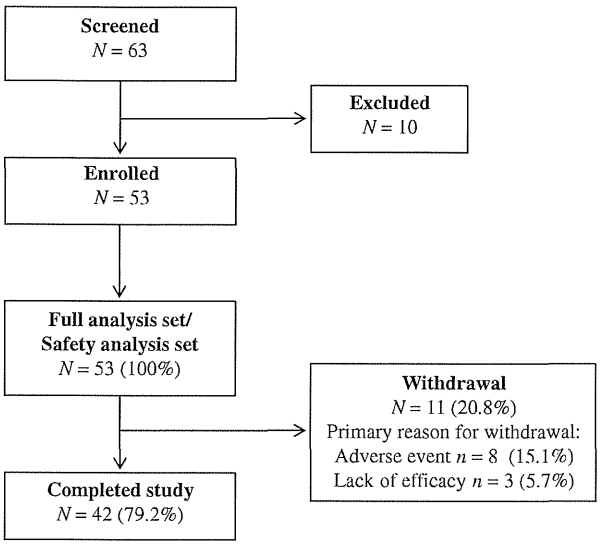

Fig. 1 Patient disposition

μL, >40 to <60 × 10^4/μL, <60 × 10^4/μL, ≤40 × 10^4/μL) and the proportion of each subgroup who achieved a response or normalization in their platelet counts was analyzed. The safety, tolerability, and utilization of anagrelide were also evaluated.

Post hoc subanalyses

Further analyses were carried out on the baseline characteristics to determine whether there were any trends in the efficacy and safety data.

Statistical methods

The study was not formally powered, but 60 patients were required to be enrolled to ensure that at least 50 would receive anagrelide treatment. It was planned that at least 10 patients would be required for each of the ≥60 × 10^4/μL and <60 × 10^4/μL platelet count baseline groups. These sample sizes were deemed large enough to give reasonably robust estimates on which to be able to draw conclusions. The efficacy variables were summarized using descriptive statistics, including the number and proportion of patients, together with a two-sided 95 % confidence interval.

Results

Study population

Of 63 patients screened, 53 met the inclusion criteria and were enrolled (Fig. 1). These patients met the WHO criteria for the diagnosis of ET (biopsy data were not collected as these were not a requirement of the protocol). Eleven patients (20.8 %) did not complete the study; the primary reasons for withdrawal were either due to an AE (n = 8; 15.1 %) or lack of efficacy (n = 3; 5.7 %). All 53 patients were included in the full analysis and safety analysis sets, which consisted of 30 females (56.6 %) and 23 males (43.4 %) with a median age of 66.0 years (range 36–86 years) (Table 1). The majority of patients were intolerant (n = 34; 64.2 %) to their previous or current CRT, rather than refractory (n = 19; 35.8 %) (Table 1).

Table 1 Baseline patient characteristics (safety set)

Characteristic	N = 53
Age (years)	
Median (range)	66.0 (36–86)
Age category, n (%)	
<50 years	12 (22.6)
50–59 years	5 (9.4)
60–69 years	18 (34.0)
70–79 years	17 (32.1)
≥80 years	1 (1.9)
Sex, n (%)	
Male	23 (43.4)
Female	30 (56.6)
Reason for stopping previous or current CRT (before entering the study), n (%)	
Refractory to CRT	19 (35.8)
Intolerant to CRT	34 (64.2)
Prior CRT, n (%)	
Anagrelide hydrochloride	2 (3.8)
Bulsulfan	2 (3.8)
Hydroxycarbamide	53 (100.0)
Interferon-α	2 (3.8)
Mercaptopurine	1 (1.9)
Ranimustine	6 (11.3)
Vincristine sulfate	1 (1.9)
Patients taking concomitant hydroxycarbamide up to month 1, n (%)	27 (50.9)
Time since ET diagnosis, years	
Median (range)	6.88 (0.12–29.06)
Baseline hemoglobin (g/dL)	
Mean (SD)	12.1 (1.91)

CRT cytoreductive therapy; *ET* essential thrombocythemia; *SD* standard deviation

Efficacy

The median length of anagrelide treatment was 358.0 days and the median daily dose was 1.904 mg/day (range 0.58–5.48 mg/day) (Table 2). The maximum final dose

Table 2 Anagrelide exposure (safety set)

Exposure	$N = 53$
Length of exposure, days	
Mean (SD)	305.2 (117.03)
Median (range)	358.0 (13–367)
Total dose (g)	
Mean (SD)	0.657 (0.4368)
Median (range)	0.535 (0.02–2.01)
Average daily dose (mg/day)	
Mean (SD)	2.126 (1.0482)
Median (range)	1.904 (0.58–5.48)
Length of exposure category, *n* (%)	
<3 months	7 (13.2)
3 to <6 months	2 (3.8)
6 to <9 months	1 (1.9)
9 to <12 months	1 (1.9)
12 months (complete)	42 (79.2)
Compliance, *n* (%)	
<80 %	0
80–90 %	1 (1.9)
90–100 %	45 (84.9)
100–110 %	7 (13.2)
110–120 %	0
>120 %	0

SD standard deviation

administered on the study was 7 mg/day, and was administered to one patient. Thirty-six patients (67.9 %) responded (platelet count of $<60 \times 10^4/\mu L$ for consecutive visits ≥4 weeks following ≥3 months of anagrelide treatment) while on anagrelide treatment (Fig. 2). The median time to response was 98.5 days. Twenty-four patients (45.3 %) achieved normalization in platelet count [platelet count of $\leq 40 \times 10^4/\mu L$ for consecutive visits ≥4 weeks following ≥3 months of anagrelide treatment (Fig. 2)], with a median time to normalization of 274.0 days. Half of the patients achieved a 50 % reduction in their platelet counts (Fig. 2). Mean platelet counts and mean study drug dose over time are presented in Fig. 3. Of the 47 patients who had a baseline platelet count of $\geq 60 \times 10^4/\mu L$, 32 (68.1 %) achieved a response and 21 (44.7 %) achieved normalization in their platelet counts. Of the six patients who had a baseline platelet count of $<60 \times 10^4/\mu L$, four (66.7 %) responded and three (50.0 %) achieved normalization. One patient had a baseline platelet count $\leq 40 \times 10^4/\mu L$ and was considered as not achieving a response or normalization as they did not complete at least 3 months of treatment (withdrew after 63 days). Five patients had baseline platelet counts of >40 to $<60 \times 10^4/\mu L$; four (80 %) of these responded and three (60.0 %) achieved platelet normalization.

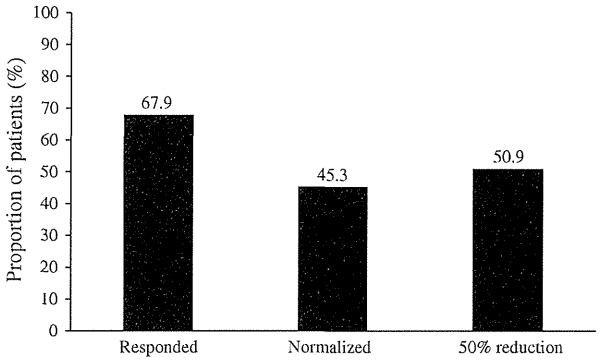

Fig. 2 Proportion of patients with a platelet count response ($<60 \times 10^4/\mu L$), platelet count normalization ($\leq 40 \times 10^4/\mu L$), and 50 % reduction in platelet count compared with their baseline values

The post hoc subanalyses explored any trends in the efficacy of anagrelide in terms of the baseline characteristics of the patients. Notable differences were observed in the mean baseline platelet counts in the following subgroups: refractory patients ($121.6 \times 10^4/\mu L$) compared with the intolerant patients [$91.3 \times 10^4/\mu L$ (Fig. 3b)]; patients who continued to receive concomitant HC for the first month ($91.0 \times 10^4/\mu L$) compared with those who switched directly to anagrelide treatment [$113.8 \times 10^4/\mu L$ (data not shown)]; patients with a baseline platelet count of $>100 \times 10^4/\mu L$ ($141.4 \times 10^4/\mu L$) compared with those with a baseline platelet count $\leq 100 \times 10^4/\mu L$ [$74.3 \times 10^4/\mu L$ (data not shown)]. Despite these differences at baseline, each of the subgroups analyzed achieved mean platelet counts of $<60 \times 10^4/\mu L$ at the 12-month visit. Also, variations were observed in the median time taken to achieve response in some subgroups: refractory patients (92.0 days) compared with the intolerant patients [124.0 days (Fig. 3b)]; patients with a baseline platelet count of $>100 \times 10^4/\mu L$ (144.5 days) compared with those with a baseline platelet count $\leq 100 \times 10^4/\mu L$ (92.0 days (data not shown)]; patients with a previous history of thrombohemorrhagic events (152.5 days) compared with patients without a previous history of thrombohemorrhagic events (92.0 days). In addition to this, it was also observed that the response rate varied in some subgroups: refractory patients (78.9 %) compared with intolerant patients (61.8 %).

Safety

All patients reported treatment-emergent adverse events (TEAEs) (Table 3), the majority of which were either mild or moderate in severity ($n = 46$; 86.8 %). The most common were anemia [$n = 25$; 47.2 % (mild 21; moderate 4)], headache [$n = 24$; 45.3 % (mild 19; moderate 3; severe 2)], palpitations [$n = 20$; 37.7 % (mild 19; severe 1)], and

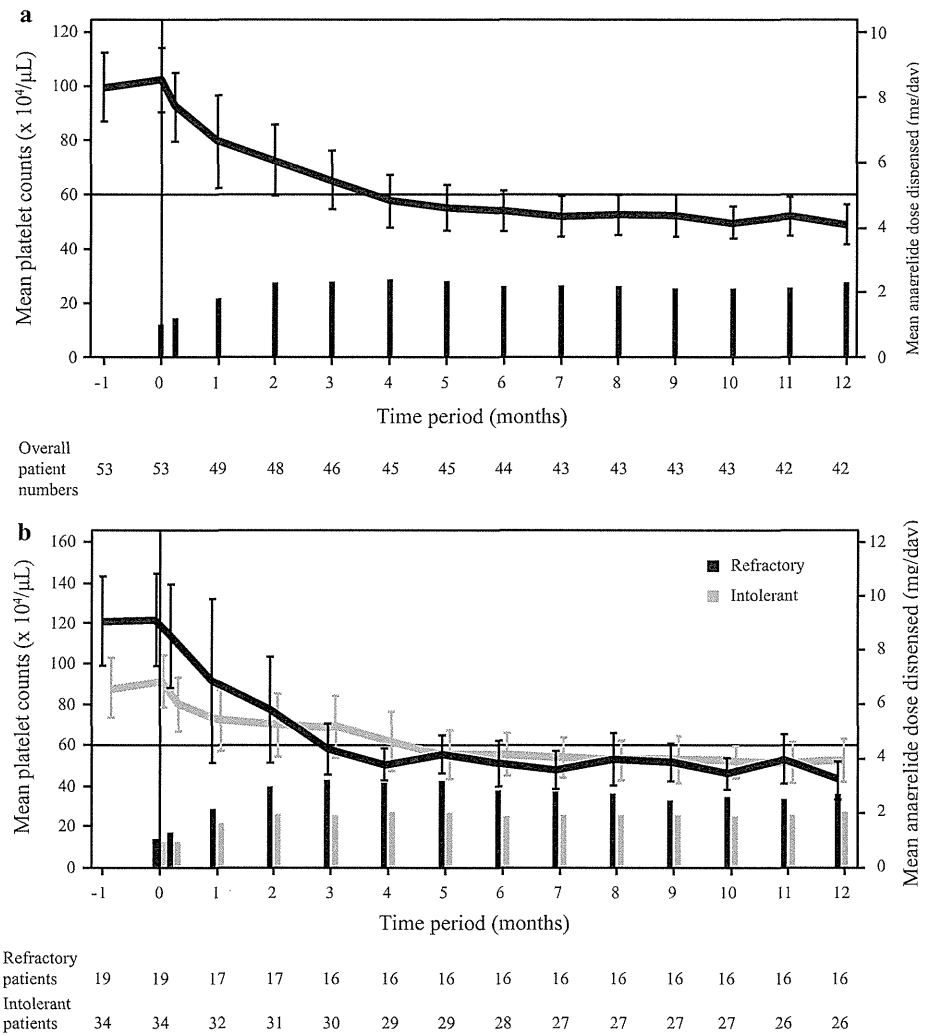

Fig. 3 **a** Mean platelet counts (and 95 % confidence intervals) and mean anagrelide dose over time (full analysis set) and **b** mean platelet counts and mean anagrelide dose over time in patients who were refractory (black line and bars) or intolerant (gray line and bars) to previous or current cytoreductive therapy. *Note* Baseline data are presented at 0 months. Overall patient numbers represent the number of patients that were still in the study at that time point. Patient numbers at Week 1 are not shown as no patients had discontinued the study by this time point

diarrhea [$n = 17$; 32.1 % (all mild)]. Mean hemoglobin levels were 12.1 g/dL [standard deviation (SD) 1.91] upon study entry (Table 1) and they declined to 10.7 g/dL (SD 1.58) by the end of the study. Two patients experienced Common Terminology Criteria for Adverse Events (CTCAE) grade 3 headache, and one patient experienced CTCAE grade 3 palpitations. No patients experienced CTCAE grade 4 or 5 adverse events. A total of 25 serious TEAEs were reported in 15 patients (28.3 %), including two patients with cerebral infarction and one patient with lacunar infarction (Table 3). Of the 25 serious TEAEs, cytogenetic abnormality, pneumonia ($n = 2$ each; 3.8 %), palpitations, visual impairment, melena, edema, altered state of consciousness, headache, interstitial lung disease, and cerebral infarction ($n = 1$ each; 1.9 %) were considered treatment-related. In two patients, transformation to leukemia was assumed and the patients were tested for other cytogenetic abnormalities in addition to the *BCR-ABL* fusion signals. Trisomy 8 was detected in one patient; however, *BCR-ABL* fusion signals were not detected in peripheral cells by fluorescence in situ hybridization. The other patient had a cytogenetic abnormality on chromosome 46. Myeloblasts were detected in peripheral blood and bone marrow, but no progression to leukemia was observed. A total of 16 TEAEs leading to dose discontinuation were reported in nine patients (17.0 %); all occurred in one patient each, except palpitations ($n = 2$; 3.8 %) and headache ($n = 3$; 5.7 %). Treatment was discontinued within 14 days of TEAE onset for nearly all patients ($n = 8/9$). One patient discontinued treatment 23 days after the first TEAE onset, but all four TEAEs experienced by this patient were reported as mild. The primary reason given for withdrawal in one of these nine patients (who reported an event of anemia that led to dose discontinuation) was given as lack of efficacy. Clinically significant abnormalities in electrocardiograms (ECG) were reported

Table 3 Treatment-emergent adverse events reported by ≥ 10 % of patients and treatment-emergent serious adverse events (safety set)

Preferred term, *n* (%)	*N* = 53
Treatment-emergent adverse events reported by ≥ 10 % of patients	
Anemia	25 (47.2)
Headache	24 (45.3)[a]
Palpitations	20 (37.7)[a]
Diarrhea	17 (32.1)
Edema peripheral	14 (26.4)
Nasopharyngitis	12 (22.6)
Pyrexia	10 (18.9)
Fatigue	9 (17.0)
Gamma-glutamyltransferase increased	7 (13.2)
Gingival bleeding	7 (13.2)
Back pain	6 (11.3)
Blood alkaline phosphatase increased	6 (11.3)
Contusion	6 (11.3)
Dyspnea	6 (11.3)
Epistaxis	6 (11.3)
Hypoesthesia	6 (11.3)
Treatment-emergent serious adverse events	
Cytogenetic abnormality	2 (3.8)
Pneumonia	2 (3.8)
Cerebral infarction	2 (3.8)
Leukocytosis	1 (1.9)
Splenomegaly	1 (1.9)
Palpitations	1 (1.9)
Visual impairment	1 (1.9)
Colonic polyp	1 (1.9)
Gastric ulcer	1 (1.9)
Melena	1 (1.9)
Edema	1 (1.9)
Pyrexia	1 (1.9)
Pyelonephritis	1 (1.9)
Laceration	1 (1.9)
Angioimmunoblastic T cell lymphoma	1 (1.9)
Prostate cancer	1 (1.9)
Altered state of consciousness	1 (1.9)
Headache	1 (1.9)
Hematuria	1 (1.9)
Interstitial lung disease	1 (1.9)

[a] Two patients experienced Common Terminology Criteria for Adverse Events (CTCAE) grade 3 headache, and one patient experienced CTCAE grade 3 palpitations. No patients experienced CTCAE grade 4 or 5 adverse events

in four patients: one patient had abnormalities in their ECG on four occasions, while the other three patients had one occurrence of an ECG abnormality. Two patients experienced QT prolongation but these were not considered clinically significant on an accompanying ECG. There were no deaths reported during the study.

The post hoc subanalyses explored any differences there may have been in the safety data in the different baseline characteristic groups. The only notable difference observed was a higher incidence of anemia in refractory patients [12/19 (63.2 %); median dose 2.622 mg/day] compared with intolerant patients [13/34 (38.2 %); median dose 1.695 mg/day]. Refractory patients entered the study with a mean baseline hemoglobin level of 12.7 g/dL, which decreased to 10.9 g/dL (change from baseline −1.8 g/dL) by the end of the study. Intolerant patients entered the study with a mean baseline hemoglobin level of 11.8 g/dL, and these values decreased to 10.6 g/dL (change from baseline −1.2 g/dL) by the end of the study. Thus it should be noted that hemoglobin levels, particularly in the intolerant patients, were low upon study entry and that some patients may have been considered anemic at study entry.

Discussion

In Japan, HC is licensed as a first-line treatment for patients with ET; however, currently there is no product specifically licensed for second-line treatment of the disease. Anagrelide is licensed in Europe as a second-line treatment for patients with ET who are intolerant or refractory to their current CRT [6]. In this Phase III, open-label, single-arm study, high-risk Japanese patients with ET who were intolerant or refractory to their first-line CRT were initiated on anagrelide therapy. Over the 12-month study period, anagrelide treatment reduced platelet counts, and demonstrated a safety profile consistent with the European SPC [6] and US prescribing information [10].

In this study, 67.9 % of patients achieved a platelet response ($<60 \times 10^4/\mu L$) with a median time to response of 98.5 days (approximately 3.2 months), 45.3 % achieved normalization ($\leq 40 \times 10^4/\mu L$) with a median time to normalization of 274.0 days, and half of the patients achieved a 50 % reduction in their platelet counts following second-line treatment with anagrelide. Studies using anagrelide as a first-line treatment in non-Japanese patients, including those in Europe, Australia, and Singapore, have revealed similar response rates [11, 12]. In the PT-1 study, patients who received anagrelide plus aspirin as a first-line therapy reached a platelet response 3–8 months after trial entry [12]. In the Anagrelide vs. Hydroxyurea in Patients with Essential Thrombocythaemia (ANAHYDRET) study, high-risk patients with newly diagnosed or treatment-naïve ET who received anagrelide first-line monotherapy reached a platelet response by 3 months of treatment [11]. In the present study, it should be noted that more refractory patients (78.9 %) reached a platelet response than the

intolerant patients (61.8 %), and the refractory patients reached a response quicker than the intolerant patients (92.0 vs. 124 days, respectively). Both these phenomena could be related to the fact that the refractory patients received a higher median daily dose of anagrelide compared with the intolerant patients (2.622 vs. 1.695 mg/day). It is also interesting to note that up-titration of anagrelide was more pronounced in the refractory patients compared with the intolerant patients, thus possibly explaining the more rapid time to response in this patient group. However, no firm conclusions can be made due to the small numbers of patients in each of the subgroups.

The most frequently reported TEAEs in this study were anemia, headache, palpitations, and diarrhea. These are all commonly or very commonly reported anagrelide-related adverse reactions according to the European SPC [6] and US prescribing information [10], and are consistent with the results from the recent Phase I/II study in treatment-naïve Japanese patients with ET [9] and a post-marketing surveillance study in Korea [13]. There were two events of cerebral infarction, and one event of lacunar infarction on the study, which were possibly related to the thrombotic events that underlie the pathophysiological process of ET. The rate of withdrawal due to an AE observed in this study (15.1 %) is comparable to that reported in the PT-1 (27.2 %) and ANAHYDRET (5.7 %) trials [11, 12]; however, discontinuation definitions differ between studies.

In the present study, 45.3 % of patients entered the study with a history of anemia, and mean baseline levels of hemoglobin were relatively low (12.1 g/dL). This could be due to a number of factors including diet-related iron deficiency, which has been recognized in Japanese individuals [14], and it is also known that patients receiving long-term HC can experience anemia, due to its myelosuppressive effects [15]. TEAEs of anemia were reported in almost half [25/53 (47.2 %)] of the patients in the current study, but all cases were either considered mild or moderate in severity. Mean hemoglobin levels at the end of the study were 10.7 g/dL, representing a change from baseline of −1.4 g/dL. Anemia is a common side effect of anagrelide use (affecting 1–10 users in 100 [6]). Similar incidences of anemia have been reported in treatment-naïve Japanese patients with ET [5/12 (41.7 %)] [9]. However, in two previous, large-scale anagrelide studies in non-Japanese patients with ET, the incidence of anemia was reported at 9.0 % [11] and 7.9 % (iron-deficiency anemia and other anemia [12]). While the reasons for the high incidence of anemia reported in the current study are not fully understood, it can be hypothesized that several factors may have influenced this phenomenon, including the fact that no specific criteria for diagnosing anemia were included in the study protocol, and such AEs were only based on investigators' judgment.

Patients were treated in accordance with the European SPC for anagrelide [6] and received an initial starting dose of 1.0 mg/day for 1 week, divided into two separate doses of 0.5 mg. Doses were then titrated thereafter in order to find the clinically effective dose for each patient. In this study, the median daily dose was 1.90 mg/day (range 0.58–5.48 mg/day), which is within the European SPC [6] and the US prescribing information (for adult patients with thrombocythemia, secondary to myeloproliferative disorders) [10] recommended maintenance doses (1–3 and 1.5–3 mg/day, respectively). The median daily dose observed in this study is also similar to the dose reported in the recent Evaluation of Xagrid Efficacy and Long-term Safety (EXELS) study in Europe (1.5 mg/day) [16, 17]. The dosing observed in this present study further supports evidence from a previous study in treatment-naïve patients with ET [9] that Japanese-specific anagrelide dosing regimens are not required.

There was a broad range of anagrelide doses administered in the present study (0.5–7.0 mg/day). These are similar to those reported in the previous Japanese study in treatment-naïve patients with ET (0.5–5.5 mg/day) [9] and the EXELS European study (0.5–6.0 mg/day) [17]. These results indicate that individualized treatment regimens are required to ensure each patient receives the lowest effective dosage required to reduce and maintain platelet counts.

A limitation of the present study is that a comparator arm was not included because there was no approved second-line treatment for ET in Japan. This limits our ability to draw firm conclusions regarding our results; however, overall these data are in line with previously reported data from Caucasian patients and support the use of anagrelide in Japanese patients with ET. In addition, although a diagnosis of ET according to the WHO criteria was required for enrollment on the study, it was not reconfirmed at study entry, i.e. further bone biopsies were not carried out as it was considered too burdensome for the patient.

In conclusion, these data demonstrate that anagrelide effectively reduces platelet counts in high-risk Japanese patients with ET who are intolerant or refractory to their previous CRT, supporting the use of anagrelide as a second-line therapy for ET in this patient population. Dosing of anagrelide was comparable to previous studies of Caucasian patients, although it should be noted that individualized dosing is required to ensure patients achieve a maximum platelet response at the lowest effective dose. Anagrelide's safety profile in Japanese patients is consistent with the European SPC and US prescribing information.

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Conflict of interest YK has provided consultancy for a clinical trial. YM has provided consultancy to Eisai and Kyowa Hakko Kirin, received payment for lectures including service on speakers' bureaus from GlaxoSmithKline, Kyowa Hakko Kirin, and Shire, and has a patent pending for a screening model of drugs for megakaryopoiesis. PW is an employee of, and holds stocks/stock options in, Shire Pharmaceuticals. JS is a former contractor of Shire Pharmaceuticals. HA is an employee of Shire Pharmaceuticals. SO has received honoraria from Pfizer, Bristol-Myers Squibb, and Novartis; he has also received donations from Kyowa Hakko Kirin and Chugai.

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Case Report

Postoperative Atypical Hemolytic Uremic Syndrome Associated with Complement C3 Mutation

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Atypical hemolytic uremic syndrome (aHUS) can be distinguished from typical or Shiga-like toxin-induced HUS. The clinical outcome is unfavorable; up to 50% of affected patients progress to end-stage renal failure and 25% die during the acute phase. Multiple conditions have been associated with aHUS, including infections, drugs, autoimmune conditions, transplantation, pregnancy, and metabolic conditions. aHUS in the nontransplant postsurgical period, however, is rare. An 8-month-old boy underwent surgical repair of tetralogy of Fallot. Neurological disturbances, acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia developed 25 days later, and aHUS was diagnosed. Further evaluation revealed that his complement factor H (CFH) level was normal and that anti-FH antibodies were not detected in his plasma. Sequencing of his CFH, complement factor I, membrane cofactor protein, complement factor B, and thrombomodulin genes was normal. His ADAMTS-13 (a disintegrin-like and metalloprotease with thrombospondin-1 repeats 13) activity was also normal. However, he had a potentially causative mutation (R425C) in complement component C3. Restriction fragment length polymorphism analysis revealed that his father and aunt also had this mutation; however, they had no symptoms of aHUS. We herein report a case of aHUS that developed after cardiovascular surgery and was caused by a complement C3 mutation.

1. Introduction

Thrombotic microangiopathy (TMA) is a clinical pathologic disorder characterized by the presence of microthrombi in multiple organ systems, including the kidneys and brain. Peripheral blood smears show fragmented red blood cells and thrombocytopenia [1]. TMA forms the pathophysiologic basis of several clinical syndromes, including hemolytic uremic syndrome (HUS); thrombotic thrombocytopenic purpura (TTP); and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome [2]. HUS and TTP were previously considered to be part of a single spectrum of TMA. However, recent research and chemical analysis of patients' serum have indicated that HUS and TTP are separate entities with distinct pathogenetic processes [1]. Although HUS occurs infrequently, it is the most common TMA in the pediatric population. About 90% of cases in children are associated with Shiga-like toxin produced mainly by *Escherichia coli* 0157:H7, *Shigella dysenteriae* type 1, and other pathogens [3]. Atypical HUS (aHUS), which can be distinguished from typical or Shiga-like toxin-induced HUS, may occur secondary to infections, malignancies, drugs, pregnancy, and autoimmune disease [3].

aHUS can be sporadic or familial. More than half of patients with aHUS exhibit genetic loss-of-function mutations of regulators (complement factor H (CFH), complement factor I (CFI), membrane cofactor protein (MCP),

and thrombomodulin (THBD)) [4–7]. Additionally, gain-of-function mutations of key complement component C3 and complement factor B (CFB) [8, 9] have been found to predispose to aHUS.

However, the pathophysiology of TTP, which has been largely elucidated in recent years, involves an imbalance between the levels of von Willebrand factor and its cleaving protease, a disintegrin-like and metalloprotease with thrombospondin-1 repeats 13 (ADAMTS-13). This imbalance leads to the presence of large multimers of von Willebrand factor, which then bind platelets and form thrombi in various organs. Low activity of the cleaving protease has been noted in adults with antibodies to ADAMTS-13 [10]. Congenital defects in the ADAMTS-13 gene lead to low levels of the protease; this is the most common cause of TTP in children.

Multiple conditions have been associated with aHUS, including infections, certain drugs, autoimmune conditions, transplantation, pregnancy, and metabolic conditions [11, 12]. However, aHUS occurring in the nontransplant postsurgical period has rarely been reported.

2. Case Presentation

An 8-month-old boy was referred to the Department of Pediatric Cardiovascular Surgery in our center for surgical repair of tetralogy of Fallot. His initial preoperative blood count and complete chemistry results were normal. His complement levels were not examined. The patient was transferred to the pediatric intensive care unit for observation and further management. On postoperative day 1, he was found to have anemia with a hemoglobin level of 10.6 g/dL and thrombocytopenia with a platelet count of 21,000/mm^3 (Table 1(a)). The patient's prothrombin time-international normalized ratio (PT-INR) and activated partial thromboplastin time (APTT) were prolonged at 1.47 and 30.10 seconds, respectively. The patient was diagnosed with disseminated intravascular coagulation (DIC) and treated with fresh frozen plasma (FFP) and a platelet transfusion. Macrohematuria developed on postoperative day 8, and FFP and platelet transfusions were performed repeatedly to control his bleeding. However, his renal function and consciousness level only temporarily improved; disturbance of consciousness and renal dysfunction redeveloped on postoperative days 16 to 24 (Figure 1).

On postoperative day 25, physical examination demonstrated a blood pressure of 90/57 mmHg and a pulse of 105/min. Laboratory examination showed only mild anemia with a hemoglobin level of 11.5 g/dL as well as thrombocytopenia with a platelet count of 13,000/mm^3. Schizocytes were observed in the patient's blood smear, and a low haptoglobin level (<10 mg/dL) was noted. The patient's PT-INR and APTT were prolonged at 2.33 and 45.0 seconds, respectively. Blood chemistry results disclosed renal failure, with a creatinine and blood urea nitrogen level of 1.18 and 108.00 mg/dL, respectively. His complement levels (reference ranges) were as follows: C3, 40 mg/dL (69–128 mg/dL); C4, 12.7 mg/dL (14–36 mg/dL); and CH50, 19.5 U/mL (25–50 U/mL). His other blood chemistry data were as follows: total protein, 4.9 g/dL; albumin, 2.8 g/dL; total bilirubin, 2.07 mg/dL; serum aspartate aminotransferase, 126 IU/L; and serum alanine aminotransferase, 8 IU/L (Tables 1(a) and 1(b)). Urinalysis showed proteinuria and hematuria. The patient had neither diarrhea nor bloody stool. Stool culture results were negative for *E. coli* O157. According to the above data and the patient's neurological disturbances, acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, TMA (most likely aHUS) was considered as the working diagnosis. On postoperative day 26, brain CT was performed to identify the cause of the persistent disturbance of consciousness and showed severe, extensive brain edema. Furthermore, pupillary light reflex deficits were observed. The patient was not expected to recover without neurological sequelae. Therefore, continuous hemodiafiltration, peritoneal dialysis, and plasma transfusion were performed as conservative therapy. The patient died on postoperative day 50.

Table 1: (a) Time series of patient's laboratory data. (b) Specific data of the patient for aHUS (POD25).

(a)

Parameters (unit of measurement)	−1POD	1POD	25POD	Normal range
White blood cell (/mm^3)	14700	3700	29900	4000–9000
Hemoglobin (g/dL)	14.3	10.6	11.5	11.0–15.0
Platelet (×10^4/mm^3)	35.4	2.1	1.3	16.1–36.0
Albumin (g/dL)	4.8	3.0	2.8	3.9–4.9
AST (U/L)	73	294	126	12–29
ALT (U/L)	38	25	8	5–29
LDH (U/L)	292	1167	2147	106–220
Total bilirubin (mg/dL)	0.62	2.31	2.07	0.4–1.3
BUN (mg/dL)	9	9	108	9–21
Creatinine (mg/dL)	0.18	0.33	1.18	0.80–1.30
Haptoglobin (mg/dL)			<10	19–170
Schizocyte			+	(−)
PT-INR	1.03		2.33	1.0
APTT (s)	19.9		46.0	20–30
FDP (μg/mL)	2		42	0–5

(b)

Parameters (unit of measurement)	Results	Normal range
C3 (mg/dL)	40.0	86–160
C4 (mg/dL)	12.7	17–45
CH50 (IU/mL)	19.5	35–45
CFH gene mutation	Not detected	Not detected
CFI gene mutation	Not detected	Not detected
CFB gene mutation	Not detected	Not detected
MCP gene mutation	Not detected	Not detected
C3 gene mutation	R425C	Not detected
THBD gene mutation	Not detected	Not detected
Anti-FH antibody	Not detected	Not detected
ADAMTS-13 activity assay	Normal	Normal

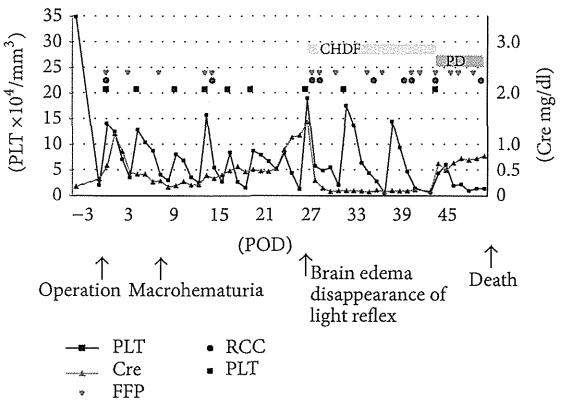

FIGURE 1: Laboratory findings during the clinical course.

Further evaluation revealed that his CFH level was normal, and a hemolytic assay using the patient's serum and that of normal controls showed no significant difference. Anti-FH antibodies were not detected in the patient's plasma. Sequencing of the CFH, CFI, MCP, CFB, and THBD genes was normal. His ADAMTS-13 activity was also normal (Table 1(b)). However, he had a potentially causative mutation (R425C) in the b-chain of C3 in exon 12. This finding confirmed that the patient had aHUS caused by a C3 gene mutation.

The patient's parents gave consent for C3 gene analysis in the patient, his elder sister, and his aunt (Figure 2(a)). Restriction fragment length polymorphism analysis confirmed that his father and aunt had this same mutation (Figure 2(b)). The patient's father and aunt had no history of any surgical procedures, although they developed the common cold at a typical frequency. Furthermore she had not become pregnant before then.

3. Discussion

We have presented a case of aHUS that developed in an infant after cardiac surgery for repair of tetralogy of Fallot. aHUS has been used to classify any HUS not caused by Shiga toxin. A variety of precipitating events have been associated with aHUS, including infections, drugs, autoimmune conditions, vaccination, malignancy, organ transplantation, pregnancy, and metabolic conditions [11, 12]. Although it is an uncommon postoperative complication, aHUS must be considered as a possible cause of acute kidney injury after surgical procedures [13]. Above all, the alternative complement pathway plays a key role in the pathogenesis of aHUS [11, 12]. Mutations in CFH account for approximately 25% of the genetic predisposition to aHUS [11, 14]. Mutations in CFI and MCP account for 5% to 10% and 10% of cases of aHUS, respectively [11, 15]. Mutations in C3 have been reported in several cohorts of patients with aHUS at a frequency of 4% to 10% [12, 16, 17].

Mutations of complement component C3 have been described more recently. C3 is cleaved to form the anaphylatoxins C3a and C3b, which are highly reactive and can bind to cell surfaces via their reactive thioester. C3b then can interact with CFB in the presence of factor D to form the alternative pathway of complement C3 convertase (C3bBb), which further cleaves C3, introducing a positive-feedback loop. Initial functional analysis showed that MCP was unable to bind to mutant C3, preventing its cleavage to iC3b [8]. Two C3 mutations that result in decreased secretion have been described, but their pathogenetic role remains uncertain. More recently, two mutations in C3 that bind to CFB with higher affinity and cause increased C3 convertase formation have been reported [18, 19]. These mutations result in increased complement activation on platelets [18] and the glomerular endothelium [19].

The underlying pathogenesis of TMA is considered to involve endothelial cell injury that results in renal arteriolar peritubular capillaries, intracapillary platelets, and fibrin-rich thrombi formation [20]. It is speculated that the pathogenesis of postoperative TTP involves massive endothelial damage during surgery [21]. In the present case, the endothelial cells might have been damaged during cardiac surgery. The presence of severe thrombocytopenia (platelet count $\leq 2.1 \times 10^4/\mu$L), anemia, and hemolytic parameters (elevated LDH and bilirubin levels) was observed immediately after surgery. These findings might be clues to the presence of aHUS that developed hyperacutely (Figure 1).

Fan et al. [22] recently reported the cause of aHUS in 10 Japanese patients. Eight cases were sporadic and the other two arose from one family. They 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 THBD (p.T500M) in 8 of the 10 patients. The patient with the n p.R425C mutation was our patient in the present report. Two mutations, p.R425C and p.S562L, are novel, and the p.I1157T mutation has been previously reported in the United States and Spain [16].

Fan et al. [22] described another patient with C3 mutation for whom surgery became the probable triggering event. The patient also had C3 p.I1157T, developed aHUS after undergoing nephrectomy at 70 years of age, and was treated with hemodialysis [23]. They stated that, in addition to the main genetic mutation, environmental factors and/or other genetic variations were likely required for the manifestation of aHUS as a secondary hit [23]. The cardiac surgery and/or presence of DIC might be secondary hits because other triggering events such as respiratory infection, diarrhea, aHUS-inducing drugs, and others were not demonstrated in our patient. Additionally, although our patient's father and aunt were affected by aHUS, they had heterozygous C3 mutation pR425C. This may be why they have not yet encountered a secondary hit.

As mentioned above, a variety of precipitating events are thought to contribute to the development of aHUS [11]. It is often reported that kidney transplantation is also a causative factor in aHUS [24]. However, there has been only one report describing the onset of aHUS initiated by a surgical procedure other than transplantation [13]. In that report, a 66-year-old woman developed renal impairment on the first day after laparoscopic hemicolectomy. aHUS is

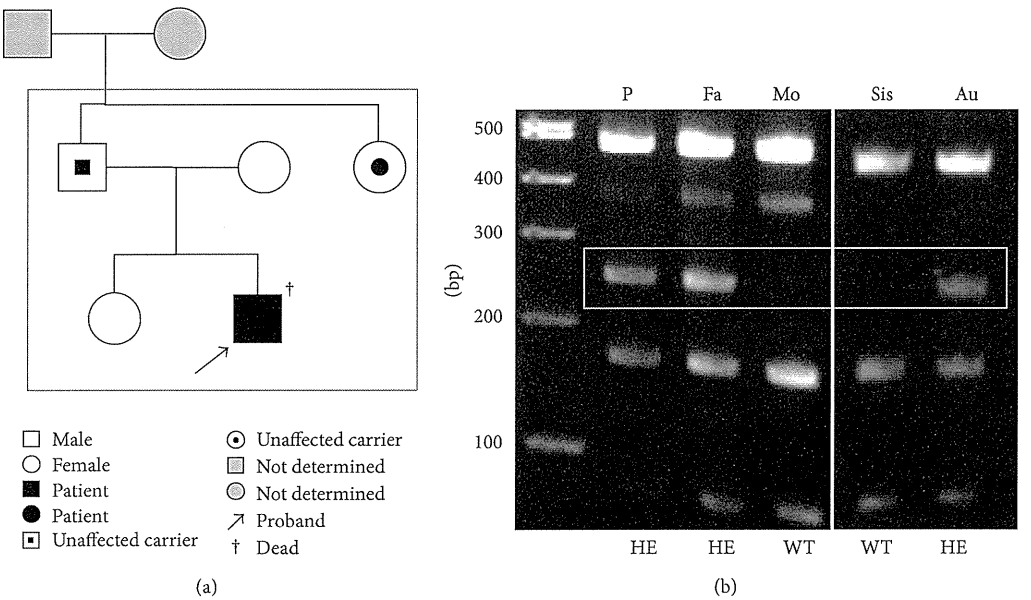

FIGURE 2: (a) The pedigree of the patient. (b) RFLP analysis. P: proband, Fa: father, Mo: mother, Sis: sister, Au: aunt, WT: wild type, and HE: heterozygote.

an uncommon postoperative complication; however, considering its different treatment modalities and poor outcomes, aHUS must be considered as a possible cause of acute kidney injury combined with thrombocytopenia and anemia after surgical procedures [13]. Conversely, TTP occurring in the nontransplantation postsurgical setting has been reported several times and has often been described after both cardiothoracic and vascular surgeries and noncardiovascular surgeries [25–28]. To the best of our knowledge, this is the first case report of postoperative aHUS due to a complement C3 mutation after nontransplantation surgery. In postsurgical patients who develop unexplained microangiopathic hemolytic anemia and thrombocytopenia, the diagnosis of aHUS should be considered and plasma exchange should be contemplated while other causes such as infection and disseminated intravascular coagulation are evaluated.

Conflict of Interests

Dr. Yoshihiro Fujimura received a grant for collaborative research from Alexion and a research grant from Takeda Medical Foundation. The other authors declare no conflict of interests regarding the publication of this paper.

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ORIGINAL ARTICLE

Analysis of patients with atypical hemolytic uremic syndrome treated at the Mie University Hospital: concentration of *C3* p.I1157T mutation

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Abstract Atypical hemolytic uremic syndrome (aHUS) is caused by abnormalities of the complement system and has a significantly poor prognosis. The clinical phenotypes of 12 patients in nine families with aHUS with familial or recurrent onset and ADAMTS13 activity of ≥20 % treated at the Mie University Hospital were examined. In seven of the patients, the first episode of aHUS occurred during childhood and ten patients experienced a relapse. All patients had renal dysfunction and three had been treated with hemodialysis. Seven patients experienced probable triggering events including common cold, influenza, bacterial infection and/or vaccination for influenza. All patients had entered remission, and renal function was improved in 11 patients. DNA sequencing of six candidate genes, identified a *C3* p.I1157T missense mutation in all eight patients in six families examined and this mutation was causative for aHUS. A causative mutation *THBD* p.D486Y was also identified in an aHUS patient. Four missense mutations, *CFH* p.V837I, p.Y1058H, p.V1060L and *THBD* p.R403K may predispose to aHUS manifestation; the remaining seven missense mutations were likely neutral. In conclusion, the clinical phenotypes of aHUS are various, and there are often trigger factors. The *C3* p.I1157T mutation was identified as the causative mutation for aHUS in all patients examined, and may be geographically concentrated in or around the Mie prefecture in central Japan.

Keywords aHUS · C3 mutation · Trigger factor · Renal failure · Thrombotic microangiopathy

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Introduction

Hemolytic uremic syndrome (HUS) [1] is characterized by the presence of microangiopathic hemolytic anemia, thrombocytopenia, renal impairment with symptoms similar to those of thrombotic thrombocytopenic purpura (TTP) [2–4]. Approximately 10 % of cases are classified as atypical due to the absence of Shiga toxin-producing bacterial infection as a trigger [5]. Compared to typical HUS, atypical HUS (aHUS) is considered to be caused by abnormalities of the complement system and has a much poorer prognosis and higher mortality, with up to half of patients progressing to end-stage renal disease [6].

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 the subsequent activation of the complement pathway [7]. When C3b binds to host cells, further activation of the complement system is stringently limited by several endogenous complement regulatory proteins present on the surface of the host cells [8]. Complement factor H (CFH) and membrane cofactor protein (MCP or CD46) are cofactors for the proteolytic degradation of C3b by complement factor I (CFI). Thrombomodulin, an endothelial anticoagulant glycoprotein encoded by *THBD*, also functions as a cofactor for CFI-mediated C3b inactivation [9]. The uncontrolled activation of the alternative pathway of the complement system plays an important role in the pathogenesis of aHUS. More than half of patients with aHUS have mutations in the genes involved in the alternative pathway of the complement system [5]. Mutations with loss-of-function of regulators (CFH, CFI, MCP and THBD) [9–12] and gain-of-function of key complement components (C3 and CFB) [13, 14] have been found to predispose patients to the development of aHUS. A normal plasma level of complement proteins does not preclude the presence of mutations in these genes. More importantly, genotype–phenotype correlations of aHUS have clinical significance in predicting renal recovery and transplant outcomes [12].

We previously reported the clinical characteristics and genetic variations of ten aHUS patients in nine family, in whom two aHUS patients in one family have been treated at the Mie University Hospital [15]. In the present study, we examined the clinical phenotypes of 12 aHUS patients in nine families, including previously reported those two patients in one family, all treated at the Mie University Hospital. We also performed the genetic analysis in six aHUS patients, in total, identified at the Mie University Hospital. Unexpectedly, we found that all six patients shared the same genetic mutation, *C3* p.I1157T missense mutation.

Materials and methods

Twelve aHUS patients in nine families, all treated at the Mie University Hospital, were investigated in this studies. We included two previously reported patients (patients JJ1 and JJ2 [15]) in one family as patients IV-1 and IV-2, respectively, because they have been treated at the Mie University Hospital. Six of the patients were sporadic and three were familial. The diagnosis of aHUS was made based on the simultaneous occurrence of microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure without Shiga toxin [16]. In addition, patients with familial or recurrent aHUS, which is associated with an ADAMTS13 activity of more than 20 % to completely exclude TTP due to ADAMTS13 deficiency, and a survival of more than 1 year, were selected in this study. The study protocol was approved by the Mie University Graduate School of Medicine and the National Cerebral and Cardiovascular Center, and written informed consent was obtained from all of the participants.

ADAMTS13 activity assay

The ADAMTS13 activity was measured using a FRETS-VWF73 peptide (Peptide Institute, Japan) according to the method reported by Kokame et al. [17, 18].

Hemolytic assay

The hemolytic assay was performed at the Department of Blood Transfusion Medicine, Nara Medical University [15]. 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 released from the red blood cells was measured by the absorbance at 414 nm [19]. The hemolysis obtained from normal plasma spiked with monoclonal antibody against CFH (200 μg IgG/ml, final) was defined as a 100 % hemolysis as the control. The result of hemolysis in patient plasma was expressed as follows; enhanced (≥50 % of the control), moderate (15–50 %) and no hemolysis (<15 %).

Mutation screening

Genomic DNA was extracted from the peripheral blood leukocytes. 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 sequenced at Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, as previously described [15]. The A of the ATG translation initiation start site was designated as

+1 position and the initial Met was denoted as +1. Multiplex ligation –dependent probe amplification analysis was used to screen the gene deletion using a commercially available kit (MLPA kit P236-A2, MRC-Holland, the Netherlands) as previously described [15].

Results

The clinical features of 12 aHUS patients in nine families are summarized in Table 1. All of the patients showed no signs for infection with Shiga toxin-producing *Escherichia coli*. Nine families were non-consanguineous with each other. The first episode of aHUS occurred during childhood (≤10 years of age) in seven patients, while five patients experienced their first episode at more than 20 years of age. Ten patients had experienced relapse, with a varying number of relapse events. All patients had renal dysfunction and three patients had been treated with hemodialysis (HD), although they were being weaned from this treatment. Three patients had central nervous symptoms. Seven patients experienced probable triggering events, such as the common cold, influenza, bacterial infection or vaccination for influenza.

The laboratory data of 12 patients with aHUS are summarized in Table 2. The platelet count was markedly reduced in all patients, with the exception of patient IV-2 and hemoglobin levels, ranging from 5.9 to 9.8 g/dl. The levels of creatinine and lactate dehydrogenase were increased in most patients with aHUS, while the total bilirubin levels were slightly increased. The plasma ADAMTS13 activity was within the range of 40–100 % in all patients. The patients were treated with plasma exchange, transfusion of fresh frozen plasma, steroid or infusion therapy or the administration of anti-platelet, anti-hypertensive or antibiotic agents. All patients had remission, in addition, the renal function improved in 11 patients and worsened in one patient (IV-2). "When patient VI-1 developed relapse, he was treated with eculizumab and his symptoms promptly improved.

Genetic analyses of six candidate genes and the gene deletion have been performed in six aHUS patients and found that all patients had a causative mutation, p.I1157T, in *C3*, and the same mutation has been previously identified in two aHUS patients in one family (patients IV-1 and IV-2) treated at the Mie University Hospital, as summarized in Table 3. A causative mutation *THBD* p.D486Y previously identified in aHUS patients in Europe and North America [7] was also identified in an aHUS patient, I-2. Gene deletion of *CFH* and *CFHRs* were not found in the aHUS patients. DNA sequencing identified additional 12 missense mutations. Among them, two rare missense

Table 1 Subjects

	Age	Sex	Age of first episode	Relapse	Outcome	Renal dysfunction	HD	CNS symptoms	Trigger for aHUS
I-1	38	F	6	6	Survive	Positive	ND	Positive	Common cold
I-2	68	F	20	2	Survive	Positive	ND	Negative	–
II	35	F	5	6	Survive	Positive	ND	Negative	Influenza
III	12	M	1	2	Survive	Positive	Weaning	Negative	Common cold, infection
IV-1	36	M	2	7	Survive	Positive	Weaning	Positive	Common cold, infection
IV-2	71	M	70	0	Survive	Positive	Weaning	Negative	–
V	38	F	21	3	Survive	Positive	ND	Positive	Common cold, vaccine[a]
VI-1	9	M	9	1	Survive	Positive	ND	Negative	Infection
VI-2	45	M	38	0	Survive	Positive	ND	Negative	–
VII	2	M	1	1	Survive	Positive	ND	Negative	–
VIII	22	F	3	7	Survive	Positive	ND	Negative	–
IX	28	M	24	7	Survive	Positive	ND	Negative	Common cold

IV-2 the father of IV-1, *HD* hemodialysis, *ND* not done, *CNS* central nerve system

[a] Vaccine for influenza

Table 2 Laboratory data of the patients with aHUS

	Platelet ($\times 10^8$/ml)	Hemoglobin (g/dl)	Creatinine (mg/dl)	LDH (U/l)	T-Bil (mg/dl)	ADAMTS13 (%)
I-1	1.9	7.4	1.7	972	1.9	76.3
I-2[a]						100
II	3.5	5.9	4.7	4485	3.5	88.8
III	2.6	5.9	4.7	4465	2.4	53.8
IV-1	2.8	6.7	10.6	1280	1.1	92.5
IV-2	9.5	9.6	8.0	398	1.2	96.0
V	3.5	9.8	1.4	928	3.5	92.5
VI-1	1.4	6.6	1.8	3160	1.4	67.5
VI-2	1.0	7.4	1.9	2850	1.8	40.0
VII	2.6	7.2	10.9	696	2.6	97.5
VIII	2.2	6.0	0.85	1098	2.2	100
IX	1.5	7.9	2.1	1780	1.9	ND

T-Bil total bilirubin, *ND* not done

[a] Previous data not available

mutations, *CFH* p.Y1058H and p.V1060L, and two low-frequency missense mutations, *CFH* p.V837I and *THBD* p.R403K, might predispose to aHUS, and the remaining seven missense mutations were likely neutral. Patient IV-1 who has *C3* p.I1157T developed aHUS at 2 year of age and experienced seven recurrences of aHUS (Table 1). He had acute renal failure and was treated with HD. Patient IV-2, a patient IV-1's father, who also has *C3* p.I1157T, developed aHUS after undergoing nephrectomy at 70 years of age and was treated with HD. Both patients and patient VI exhibited a mildly elevated hemolytic activity, however other five patients with *C3* p.I1157T did not show an elevated activity (Table 3).

Discussion

In Japan, the frequency of Shiga toxin-producing *Escherichia coli* (STEC)-HUS is approximately 40 % of all cases of thrombotic microangiopathy (TMA), according to the national questionnaire survey of TMA [20, 21], indicating that the frequency of STEC-HUS is lower in Japan than in Europe and North America. There are few methods for detecting abnormalities in the regulatory complement system. Therefore, the hematologists diagnosed the patients with suspected aHUS as having TTP. Other doctors also diagnosed the patients as having renal disease due to TMA. Therefore, there are a few reports of aHUS in Japan. In the present study, cases of fatal TMA were excluded due to the difficulty of confirming the subject's past and family history. Although all patients evaluated in the present study survived, the outcomes of aHUS were not always good. It has recently been reported that the terminal complement inhibitor eculizumab, which is approved for the treatment of paroxysmal nocturnal hemoglobin-uria, improves aHUS [22]. Eculizumab was also approved for the treatment of aHUS in Japan in September 2013. The drug binds with high affinity to human complement protein C5 and blocks the generation of proinflammatory C5a and the membrane attack complex, C5b-9 [22, 23]. "Although eculizumab is expensive, its use promptly improved the relapse in patients VI-1. Patients III, IV-1 and IV-2, who had been tread with HD, should receive eculizumab if they exhibit a relapse. Treatment with eculizumab is recommended in the acute phase in patients with mild aHUS, such as that involving C3 mutations, and as prophylaxis in those with severe aHUS, such as that involving CFH mutations [24].

In the present study, we examined the genetic abnormalities in six aHUS patients and identified the *C3* p.I1157T mutation as a causative mutation in the patients. We have previously performed the genetic analysis in aHUS patients and identified the same mutation in three patients, two of whom were referred from the Mie University Hospital [15]. Thus, as summarized in Table 3, eight aHUS patients treated at the Mie University Hospital carried the *C3* p.I1157T mutation. This finding was unexpected because our previous genetic analysis in 10 aHUS patients revealed the heterogeneous phenotype-genotype correlation [15]. The *C3* p.I1157T mutation might be geographically concentrated in or around the Mie prefecture located in central Japan.

A causative mutation *THBD* p.D486Y previously identified in aHUS patients in Europe and North America was also identified in the present study. The allele frequency of this mutation was 0.011 in the Japanese population referred from the 1000 Genomes database [25] and the recombinant mutant thrombomodulin with this mutation showed defective C3b inactivation [9]. The significance of remaining missense mutations was unknown. Both p.Y1058H and p.V1060L in the short consensus repeat-18 domain of CFH were rare mutations. They were not

Table 3 Genetic analysis of eight aHUS patients in the six families

Patients			I-1	I-2	II	III	IV-1	IV-2	V	VI
Hemolytic assay			−	−	−	−	±	±	−	±
Missense mutations	rs number	MAF								
CFH										
c.184G>A	rs800292	0.416				p.V62I			p.V62I	p.V62I
c.2509 G>A	rs55807605	0.011						**p.V837I**		
c.2808G>T	rs1065489	0.455	p.E936D (homo)	p.E936D	p.E936D (homo)	p.E936D	p.E936D (homo)	p.E936D	p.E936D	p.E936D
c.3172T>C	rs55679475	None			**p.Y1058H**	**p.Y1058H**				**p.Y1058H**
c.3178G>C	rs55771831	None			**p.V1060L**	**p.V1060L**				**p.V1060L**
CFI										
c.603A>C	rs145769028	0.028					p.R201S	p.R201S		
c.1217G>A	rs74817407	0.096				p.R406H				
C3										
c.3470T>C		None	**p.I1157T**	**p.I1157T (homo)**	**p.I1157T**	**p.I1157T**	**p.I1157T**	**p.I1157T**	**p.I1157T**	**p.I1157T**
CFB										
c.94C>T	rs12614	0.112	p.R32W	p.R32W	p.R32W		p.R32W	p.R32W (homo)		
c.95G>A	rs641153	0.073								p.R32Q
THBD										
c.1208G>A	rs41400249	0.006							**p.R403K**	
c.1418C>T	rs1042579	0.253				p.A473V			p.A473V	
c.1456G>T	rs41348347	0.011		p.D486Y						
CNV of CFH and CFHRs					Normal	Normal	Normal	Normal	Normal	

Hemolytic assay, − no hemolysis; ± moderate hemolysis. Bold and underlined: definite causative mutation [13], Bold: rare and low-frequency potentially predisposing mutation; homo, homozygote, The A in the ATG translation initiation start site is designated as the +1 position and the initial Met denotes +1

MAF minor allele frequency taken from the 1000 genome database (http://www.1000genomes.org/data), *CFH* complement factor H, *CFI* complement factor I, *C3* complement component 3, *CFB* complement factor B, *THBD* thrombomodulin, *CNV* copy number variation, *CFHRs* CFH related genes

identified in the 1000 Genomes database [25] but were found as somatic mutations in human cancers in the COSMIC database [26]. Both p.V837I in the short consensus repeat-14 domain of CFH and p.R403K in the EGF-like 4 domain of THBD were low-frequency mutations with the minor allele frequency of 0.011 and 0.006, respectively [25]. It can be assumed that, in addition to the main genetic mutation, *C3* p.I1157T, the environmental factors and/or other genetic variations are required for the manifestation of aHUS as a second hit. Rare or low-frequency missense mutations in the aHUS patients identified in the present study may predispose to the aHUS manifestation. The remaining seven missense mutations were likely neutral.

Sheep red blood cells are rich in sialic acid and are capable of binding CFH from the plasma to protect themselves against human complement attack. Therefore, the hemolytic assays are frequently used to evaluate the function of CFH-related abnormalities [18, 27]. Generally, plasma samples containing CFH mutant with mutation in the C-terminal domains or auto-antibodies against CFH would exhibit an increased hemolytic activity. In the present study, patients with the *C3* p.I1157T mutation showed negative and weak hemolytic activity, indicating that this mutation does not directly influence the hemolytic assay.

In conclusion, we found that the clinical phenotypes of aHUS patients are various, and there are often trigger factors. The *C3* p.I1157T mutation was found in eight aHUS patients examined as the causative mutation for aHUS, and would be geographically concentrated in or around the Mie prefecture in central Japan.

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A first bout of thrombotic thrombocytopenic purpura triggered by herpes simplex infection in a 45-year-old nulliparous female with Upshaw-Schulman syndrome

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Dear Sir,

Upshaw-Schulman syndrome (USS) is a congenital deficiency of ADMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs, 13) activity caused by gene mutations. ADAMTS13 specifically cleaves unusually large von Willebrand factor multimers produced in and released from vascular endothelial cells under high shear stress conditions in the microvasculature[1,2]. Thus, in the absence of ADAMTS13 activity, the uncleaved unusually large von Willebrand factor multimers are released into the circulation, causing a life-threatening systemic disease termed thrombotic thrombocytopenic purpura (TTP). Most cases of TTP are induced by acquired autoantibodies against this enzyme. USS is an extremely rare disease, and to date approximately 100 affected patients have been reported in the literature, of whom 43 are in Japan[3].

According to our experience in Japan, bouts of TTP in USS patients are triggered by various stimuli, including pregnancy, severe infection, administration of 1-deamino-8-D-arginine vasopressin (DDAVP) and drinking large amounts of alcohol. Pregnancy is the single most common trigger in female patients. In fact, in an analysis of the natural history of our 43 USS patients in Japan, we found that 26 (60%) were diagnosed during childhood (early-onset phenotype), and the remaining 17 (40%) were diagnosed after 15 years of age (late-onset phenotype). In the early-onset group, the female:male ratio was 13:13, while it was 14:3 in the late-onset group. These 14 female patients were aged between 15 and 45 years, and nine were diagnosed during pregnancy. In contrast, all three male patients had their first bouts after 45 years of age. With regards to ADAMTS13 activity, 35 patients had extremely low levels (<0.5% of normal), seven had trace amounts (0.5-0.8% of normal), and one male patient (USS-GG2) who had his first bout of TTP at 63 years of age had some activity (2.4-3.6%). Thus, one important determinant of the late-onset phenotype in USS patients is the level of ADAMTS13 activity. However, here we present the late-onset phenotype found in a middle-aged nulliparous USS female with severe deficiency of ADAMTS13 activity (<0.5% of the normal), whose first bout of TTP was triggered by an oral herpes simplex infection at the age of 45.

The proband (USS-Y3), born in Sapporo in 1960, was the first of three siblings born to non-consanguineous parents. Her parents and two brothers have had no episodes of thrombosis or excessive bleeding. Her perinatal medical history was unclear, but she did not have any exchange blood transfusions as a neonate. By the age of 3, she suffered from repeated episodes of thrombocytopenia and was diagnosed with idiopathic thrombocytopenic purpura, for which she received transfusions of fresh whole blood on a few occasions. Further details of her medical history during childhood were unavailable. Since the age of 38, her platelet count has been occasionally evaluated at a nearby hospital. The counts were almost normal (104-175×10^9/L). However, when she has a cold, her platelet count temporarily drops to less than 50×10^9/L (minimum 19×10^9/L), but normalises without any specific medical therapy. At the age of 45, she suffered from an oral herpes simplex infection complicated by thrombocytopenia (11×10^9/L), for which the antiviral aciclovir (1,000 mg/day) was prescribed. Subsequently, she has had repeated episodes of oral herpes simplex infection; therefore, she received a prescription of acyclovir for 5 months but has not had an appreciable clinical improvement. She was referred to our hospital for analysis of the cause of her thrombocytopenia. Laboratory findings on admission were as follows: thrombocytopenia (9×10^9/L), haemolytic anaemia (red cell count 1.84×10^9/L, haemoglobin 6.7 g/dL, reticulocyte 168‰, schistocytes on a peripheral smear [2+], total bilirubin 2.8 mg/dL, lactate dehydrogenase 872 IU/L, and haptoglobin <10 mg/dL), near-normal renal function (blood urea nitrogen 19 mg/dL, creatinine 1.07 mg/dL, and positive occult blood in urine), C-reactive protein 0.1 mg/dL, negative direct and indirect Coombs' tests, and normal haemostatic

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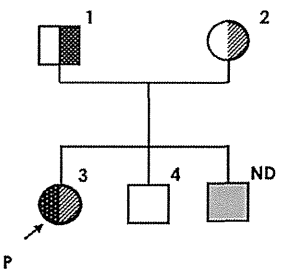
tests. She was initially treated with oral prednisolone (50 mg/day) for a diagnosis of Coombs-negative Evans syndrome, but soon thereafter her general condition worsened, and ADAMTS13 analyses were performed for diagnostic purposes.

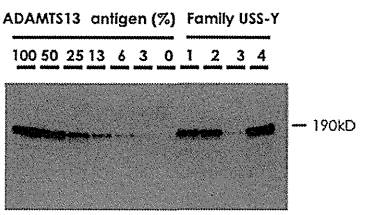
The family pedigree of this patient is shown in Figure 1A (left). The patient had very low ADAMTS13 activity (<0.5% of normal) and an absence of ADAMTS13 inhibitor (<0.5 BU/mL). Both of her parents had mild deficiencies of ADAMTS13 activity (both 34%) without its inhibitor. Plasma levels of ADAMTS13 activity in one of her younger brothers were normal (74%). Plasma levels of ADAMTS13 antigen as analysed by enzyme-linked immunosorbent assay (Figure 1B) were 2.1% of the normal control in the patient, and 25%, 22%, and 104% of normal in her father, mother, and

A

Family pedigree of USS –Y family



WB analysis



B

ADAMTS13 analysis in USS –Y family

	ADAMTS13 activity (%)	ADAMTS13 antigen (%)		*ADAMTS13 gene*		
	ELISA	ELISA	WB	Gly385	Gln448	Arg1206
Y-1	34	25	52	G/E	Q/E	R/R
Y-2	34	22	58	G/G	Q/Q	R/*
Y-3	<0.5	2.1	4	G/E	Q/Q	R/*
Y-4	74	104	102	G/G	Q/E	R/R

C

***ADAMTS13* mutations found in family Y**

Exon	Family member	Nucleotide	Amino acid	Remarks
5	Y1, Y4	c. 420 T>C	silent	common SNP
10	Y1, Y3	c. 1154 G>A	p.G385E	disintegrin-like domain
12	Y1, Y4	c. 1342 C>G	p.Q448E	common SNP
15	Y1, Y4	c. 1716 G>A	silent	common SNP
19	Y1, Y2, Y3, Y4	c. 2280 C>T	silent	common SNP
26	Y2, Y3	c. 3616 C>T	p.R1206*	CUB-1 domain
29	Y1, Y4	c. 4221 C>A	silent	common SNP

Figure 1 - The family pedigree of USS-Y is shown in Figure 1A (left). Squares and circles indicate males and females, respectively, and the arrow with P indicates the proposita. Filled symbols represent a patient of USS-Y3. The half-filled symbols represent asymptomatic carriers. Figure 1A (right) shows western blot (WB) analysis of ADAMTS13 antigen followed by anti-ADAMTS13 monoclonal antibody detection using plasma samples, according to the previous method. Note a trace amount of ADAMTS13 antigen in USS-Y3 (patient) in lane 3 of Figure 1A (right). ND indicates not determined. ADAMTS13 activity was measured by chromogenic act-enzyme linked immunosorbent assay (ELISA), and the ADAMTS13 antigen was determined by both WB and antigen-ELISA (Figure 1B). The ADAMTS13 gene mutations found in this family are shown as one-letter amino acid abbreviations (Figure 1B). The ADAMTS13 single nucleotide polymorphisms (SNP) are also shown in Figure 1C.

younger brother, respectively. Furthermore, as analysed by western blot, plasma levels of ADAMTS13 antigen (Figure 1A right and B) were 4% of the normal control in the patient, and 52%, 58%, and 102% of normal in the father, mother, and younger brother, respectively. *ADAMTS13* gene analysis revealed that the patient was a compound heterozygote for two mutations in *ADAMTS13*: p.G385E (c.1154G>A, exon 10) from her father and p.R1206* (c.3616 C>T, exon 26) from her mother. Her parents were heterozygous carriers of each of the two mutations (Figure 1B). These two mutations were not found in her younger brother. p.Q448E was reported as a single nucleotide polymorphism causing a missense mutation[4]. All mutations found in this family are shown in Figure 1C, including common single nucleotide polymorphism without amino acid substitutions. We previously reported the p.R1206X nonsense mutation in a USS-I4 patient[5]. The p.G385E missense mutation presented here is novel. Our experience indicates that the clinical phenotype of females with USS who have never been pregnant is almost indistinguishable from that of males.

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Conflict of interest disclosure

Yoshihiro Fujimura is a member of clinical advisory boards for Baxter BioScience.

All other Authors declare no conflicts of interest.

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