(ウ)治験の進捗状況について
IRB 承認後、平成 26 年 2 月 7 日付治験変更届が提出され治験開始が可能となった。希少疾患であり、現時点で被験者候補はいないが、今後も被験者のリクルートを継続する。

D) 考察

平成 25 年度の研究により、治験実施計画書が作成され、当施設において予定通り治験を開始することができた。希少疾病であり被験者のリクルートが難しい点があるが、今後、当施設の他周辺の医療機関に治験の案内をするなど引き続き工夫をしたい。

E) 結論

本研究により再発・難治性の TTP 患者に対するリツキシマブの効果と安全性を検証し、海外と同様の適応を早期に取得できることを期待する。

F) 健康危険情報

なし

G) 研究発表

Successful treatment of an elderly frail patient with acquired idiopathic thrombotic thrombocytopenic purpura under close monitoring of ADAMTS13 activity and anti-ADAMTS13 antibody titers (Transfusion and Apheresis Science : 2014)

H) 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
平成25年度　分担研究報告書

血栓性血小板減少性紫斑病に対するリツキシマブの第Ⅱ相医師主導治験

研究分担者　日笠　聡　兵庫医科大学　講師

【研究要旨】
血栓性血小板減少性紫斑病（TTP）は、溶血性貧血、血小板減少、腎障害、発熱、精神神経症状の古典的5徴候を特徴とする難治性で致死的な疾患である。TTPの治療には血漿交換療法と主にステロイドを使用した免疫抑制療法が行われるが、TTPの免疫抑制療法として抗体医薬リツキシマブの効果と安全性を評価するため、第Ⅱ相医師主導治験を開始した。

A) 研究目的
再発・難治性の血栓性血小板減少性紫斑病（TTP）に対する抗体医薬リツキシマブの効果と安全性を評価し、適応拡大の承認申請を目的とする。

B) 研究方法
(ア)治験実施計画書の作成：医薬品医療機器総合機構との薬事戦略相談により、治験実施計画書を作成する。
(イ)治験実施体制の確立：第1回班会議に参加して、他の10施設とのネットワークを形成する。院内の治験審査委員会への申請と治験開始前のセットアップミーティングを行う。

C) 研究成果
(ア)研究実施計画書の作成
平成25年9月の医薬品医療機器総合機構の薬事戦略相談の結果を受け、再発・難治性のTTP患者6名に対するシングルアーム試験をデザインした。なお、治験の対象は、成人日本人で後天性のTTP患者とする。

(イ)治験実施体制の確立
平成25年1月に慶應大学で開催した第1回班会議に、当施設から治験責任医師、治験コーディネーター、事務局担当者が参加した。平成26年1月21日の治験審査委員会で承認後、関係者を対象にした治験セットアップミーティングを行い、2月4日から治験を開始した。

(ウ)治験の進捗状況について

平成26年3月31日までに本治験の適格基準を満たした症例はなかった。

D) 考察

平成25年度の研究により、治験実施計画書が作成され、本学において予定通り治験を開始することができた。希少疾病であるため、被験者のリクルートが難しいが、できる限り症例を収集し治験を行いたい。

E) 結論

本研究により再発・難治性のTTP患者に対するリツキシマブの効果と安全性を検証し、海外と同様の適応を早期に取得できることを期待する。

F) 健康危険情報

なし

G) 研究発表

なし

H) 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
平成25年度　分担研究報告書

血栓性血小板減少性紫斑病に対するリツキシマブの第Ⅱ相医師主導治験

研究分担者　野村昌作　関西医科大学　教授

【研究要旨】
TTP は、血小板減少、細血管障害性溶血性貧血、腎機能障害、発熱、精神神経症状の古典的5 徴候で特徴付けられる血液の難病である。TTP の治療は、新鮮凍結血漿（FFP）輸注してADAMTS13 酵素補充を行い、TTP 発症予防治療が行われている場合が多い。しかし、一部の症例ではADAMTS13 活性は著減し、これらはほぼ全例ADAMTS13 インヒビター（酵素活性を抑制する自己抗体）陽性である。そのため、FFP のみの投与では不十分で、治療は血漿交換療法が第一選択となる。この際、自己抗体を減らすためステロイドの併用が一般的である。リツキシマブは、非ホジキンリンパ腫に対しての使用が保険承認されている。後天性TTP におけるリツキシマブの効果は、本邦でも2005 年から報告が増えている。国際血液学会のTTP ガイドラインにおいて、再発・難治性のTTP に対してリツキシマブは積極的に使うようグレード1で推奨されている。しかしながら、本邦において、TTP の治療薬としてリツキシマブは承認されていない。以上を踏まえて、TTP に対する抗体医薬リツキシマブの効果と安全性を評価するため、第Ⅱ相医師主導試験を開始した。

A）研究目的

再発・難治性の血栓性血小板減少性紫斑病（TTP）に対する抗体医薬リツキシマブの効果と安全性を評価し、適応拡大の承認申請を目的とする。

B）研究方法

(ア)治験実施計画書の作成：医薬品医療機器総合機構との薬事戦略相談により、治験実施計画書を作成する。

(イ)治験実施体制の確立：第1回班会議に参加して、他の10施設とのネットワークを形成する。院内の治験審査委員会への申請と治験開始前のセットアップミーティングを行う。

C）研究成果

(ア)研究実施計画書の作成
平成25年9月の医薬品医療機器総合機構の薬事戦略相談の結果

を受け、再発・難治性のTTP患者6名に対するシングルアーム試験をデザインした。なお、治験の対象は、成人日本人で後天性のTTP患者とする。

(イ)治験実施体制の確立
平成25年1月に慶應大学で開催した第1回班会議に、当施設から治験責任医師、治験コーディネーター、事務局担当者が参加した。平成26年2月院内の治験審査委員会で承認後、関係者を対象にした治験セットアップミーティングを行い、4月1日から治験を開始した。

(ウ)治験の進捗状況について
平成26年3月20日に1名の仮登録を考慮したが、ADAMTS13の測定結果よりTTPの診断基準を満たさなかったため、登録には至らなかった。

D) 考察

治験実施計画書が作成され、院内の治験審査委員会の承認を得たことから、本学において予定通り治験を開始することができた。ただし、希少疾病であるため被験者のリクルートが困難であるのが現状である。周辺の医療機関に治験の案内をするなど広報的な活動を続けたいと考えている。

E) 結論

本研究により再発・難治性のTTP患者に対するリツキシマブの効果と安全性を検証し、海外と同様の適応を早期に取得できることを期待する。

F) 健康危険情報

なし

G) 研究発表

なし

H) 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
平成25年度　分担研究報告書

血栓性血小板減少性紫斑病に対するリツキシマブの第Ⅱ相医師主導治験

研究分担者　一戸　辰夫　広島大学病院　血液内科・教授

【研究要旨】
血栓性血小板減少性紫斑病（TTP）は、ADAMTS13に対する自己抗体の産生やその他の原因による溶血性貧血、血小板減少精神神経症状を主徴とする難治性の疾患である。特に血漿交換に対する反応が不良な例や再発性の症例に対しては、現在有効な治療手段が確立していない。この度、TTPに対する抗体医薬リツキシマブの効果と安全性を評価する第Ⅱ相医師主導治験へ参加することとなった。

A）研究目的
再発・難治性の血栓性血小板減少性紫斑病（TTP）に対して、B細胞表面に発現するCD20を標的とする抗体医薬品リツキシマブの効果と安全性を評価するとともに、適応拡大の承認申請を目的とする。

B）研究方法
(ア)治験実施計画書の作成：医薬品医療機器総合機構との薬事戦略相談により、治験実施計画書を作成する。
(イ)治験実施体制の確立：第1回班会議に参加して、他の10施設とのネットワークを形成する。院内の治験審査委員会への申請と治験開始前のセットアップミーティングを行う。

C）研究成果
(ア)研究実施計画書の作成
平成25年9月の医薬品医療機器総合機構の薬事戦略相談の結果を受け、再発・難治性のTTP患者6名に対するシングルアーム試験をデザインした。なお、治験の対象は、成人日本人で後天性のTTP患者とする。

(イ)治験実施体制の確立
平成25年1月に慶應大学で開催した第1回班会議に、当施設から治験責任医師、治験コーディネーター、事務局担当者が参加した。平成26年2月院内の治験審査委員会で承認後、関係者を対象にした治験セットアップミ

ーティングを行い、3月上旬から治験を開始した。

(ウ)治験の進捗状況について
平成26年4月、TTPを強く疑う症例が当院に救急搬送されたが、対象年齢が19歳8ヶ月と治験の適格基準を満たせなかったので本登録に至らなかった。

D) 考察

平成25年度の研究により作成された治験実施計画書により、当院において予定通り治験を開始することとなった。
本治験は、県内では当院のみが参加施設ではあるものの、希少疾病であり被験者のリクルートが難しいことが予想される。対策として、広島県治験等症例集積機能向上パイロット事業に本治験の照会をかけることで、県内の主要医療機関からの症例を募るとともに、周辺の医療機関に対し、ポスター配布などの案内を行っている。これらの活動については、本年度も継続していく。

E) 結論

本研究により致死率が高いとされる再発・難治性のTTP患者に対するリツキシマブの治療の適応が取得されることで、治癒の可能性が向上することを期待する。

F) 健康危険情報

なし

G) 研究発表

なし

H) 知的財産権の出願・登録状況

なし

３．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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４．研究成果の刊行物・別刷

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

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ABSTRACT

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

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Patients

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

2.2. Complement analyses

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

2.3. ADAMTS13 activity assay

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

2.4. Hemolytic assay

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

2.5. Autoantibody against CFH

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

2.6. Mutation screening

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

2.7. Restriction fragment length polymorphism (RFLP) analysis

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

2.8. Screening for gene deletions

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

3. Results

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

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

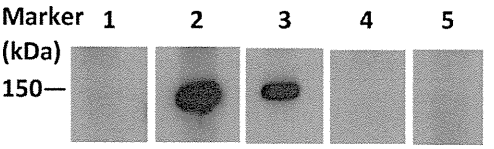

Fig. 1. Detection of CFH autoantibody in family GG. Purified CFH was electrophoresed on a 5% SDS–polyacrylamide gel and transferred to the polyvinylidene fluoride membrane. The membrane was cut into 0.5-cm wide strips and incubated with the diluted plasma sample. Horseradish peroxidase-labeled goat anti-human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

3.1. Patient X1

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

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

3.2. Patient AA1

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

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

3.3. Patient CC1

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

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

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

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

3.4. Patient DD1

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

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

3.5. Patient FF1

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

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

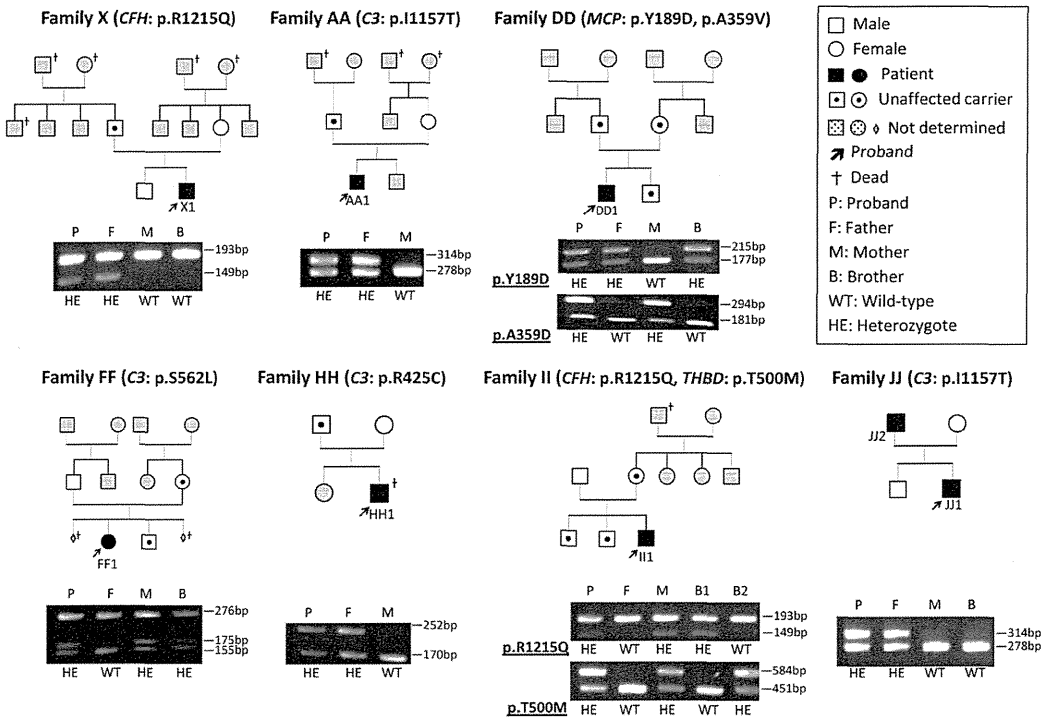

Fig. 2. Family pedigree of 8 patients with aHUS carrying causative or potentially causative mutations. Restriction fragment length polymorphism (RFLP) analyses of causative or potentially causative mutations are shown. The wild-type (WT) and heterozygote (HE) are distinguished by the electrophoretogram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

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

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

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

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

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

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

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

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

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

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

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

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

3.6. Patient GG1

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

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

3.7. Patient HH1

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

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

3.8. Patient II1

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

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

3.9. Patients JJ1 and JJ2

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

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

4. Discussion

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

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

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

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

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

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

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

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

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