analysis together revealed that the C and S domains in ADAMTS13 are necessary for strong binding to Lys-Pg. Because the M domain of ADMATS13 has two glycosylation sites (*28*), doublets of MD-FLAG might be caused by the difference of carbohydrate. Alternatively, we cannot exclude the possibility of limited proteolysis of MD-FLAG.

ADAMTS13-binding region in Lys-Pg

Elastase has been used for the domain isolation of Pg (*23*), and the resulting fragments containing kringle 1 and 4 domains can bind to lysine-Sepharose. We digested Pg with elastase and unbound and bound fractions for lysine-Sepharose were obtained. Lys-Pg, elastase-digested Lys-Pg and lysine-Sepharose unbound and bound fractions of elastase-digested Lys-Pg were subjected to SDS–PAGE (Fig. 4A) for the far-western blotting using ADAMTS13 as the ligand (Fig. 4B). As a result, ADAMTS13 bound to three bands: Lys-Pg, a 40-kDa fragment and a 32-kDa fragment (Fig. 4B, right). N-terminal sequence analysis revealed that the lysine-Sepharose bound 40-kDa fragment was the kringle 4 and 5 and protease domains (K4-K5-P) and the lysine-Sepharose unbound 32-kDa fragment was mini-Pg, which consists of the kringle 5 and protease domains (K5-P). Thus, we concluded that ADAMTS13 bound to mini-Pg but not to the kringle 1–4 domains. This was compatible with the result of the yeast two-hybrid screen that the positive clone contained C-terminal 150 residues of the protease domain of Pg.

Binding of Pg to immobilized ADAMTS13 using Biacore

ADAMTS13 was immobilized on the sensor chip, and the binding of Glu-Pg or Lys-Pg to ADAMTS13 was measured by recording the changes in surface plasmon resonance upon injection of Pgs using Biacore. We observed that Lys-Pg bound to immobilized ADAMTS13 in the dose-dependent manner (Fig. 5A), whereas Glu-Pg could not significantly bind to ADAMTS13 (Fig. 5B). Lys-Pg exhibited a higher binding affinity to ADAMTS13 with a K_d of $1.9 \pm 0.1 \times 10^{-7}$ M (the mean ± SD) than Glu-Pg with a K_d of $5.5 \pm 2.7 \times 10^{-6}$ M (the mean ± SD).

Plasmin and ADAMTS13 activities in the complex

The C and S domains of ADAMTS13 were necessary for the recognition and cleavage of VWF. Therefore, the Lys-Pg binding to ADAMTS13 may affect the ADAMTS13 activity. We examined the effects of Lys-Pg on the ADAMTS13 activity. We found that Lys-Pg did not affect the ADAMTS13 activity towards the natural substrate VWF (Fig. 6A) and the synthetic

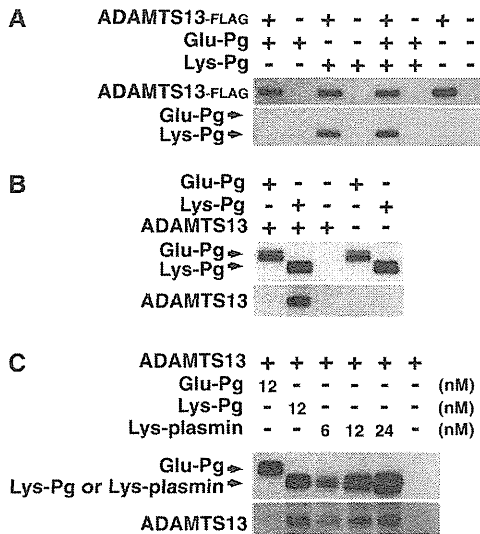

Fig. 2 Binding of ADAMTS13 to Lys-Pg. (A) Co-immunoprecipitation using anti-FLAG mAb. The culture medium containing ADAMTS13-FLAG was incubated with Glu-Pg (100 nM) and/or Lys-Pg (100 nM), and then anti-FLAG mAb-immobilized gel was added to recover bound complexes. Proteins in the complexes were subjected to SDS–PAGE for western blotting using anti-FLAG mAb or anti-Pg mAb. The result is representative of three experiments. (**B** and **C**) Co-immunoprecipitation using anti-Pg mAb. (B) Purified ADAMTS13 (6.7 nM) was incubated with Glu-Pg (12 nM) or Lys-Pg (12 nM), and then anti-Pg mAb was added. Immunocomplexes were subjected to SDS–PAGE for western blotting using anti-Pg mAb or anti-ADAMTS13 mAb WH10. (C) Binding of ADAMTS13 to active-site inhibited plasmin. ADAMTS13 (6.7 nM) was first incubated with Glu-Pg (12 nM), Lys-Pg (12 nM) and APMSF-treated plasmin (6, 12 and 24 nM) and then incubated with anti-Pg mAb. The immunocomplexes were analysed as described in (B). The result is representative of three experiments.

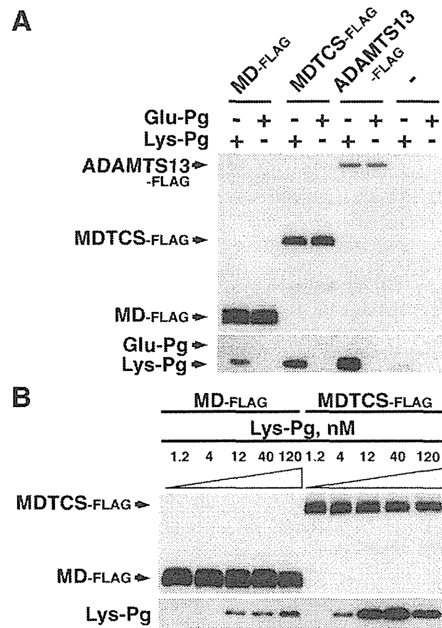

Fig. 3 Binding of ADAMTS13 and its truncated mutants to Glu-Pg or Lys-Pg. (A) Binding of MD-FLAG, MDTCS-FLAG and ADAMTS13-FLAG to Glu-Pg and Lys-Pg. The culture medium containing MD-FLAG, MDTCS-FLAG or ADAMTS13-FLAG was incubated with Glu-Pg (35 nM) or Lys-Pg (35 nM). The complex was immunoprecipitated and then probed with anti-FLAG mAb or anti-Pg mAb. The result is representative of three experiments. (**B**) Dose-dependent binding of Lys-Pg to MD-FLAG and MDTCS-FLAG. Lys-Pg (1.2, 4, 12, 40, 120 nM) was incubated with the culture medium containing MD-FLAG or MDTCS-FLAG. The complex was analysed as described in (A). The result is representative of three experiments.

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substrate FRETS-VWF73 (Fig. 6B). The plasmin activity was also not affected by ADAMTS13 even in the 10-fold molar excess of plasmin concentration (Fig. 6C).

Discussion

In this study, we have demonstrated that ADAMTS13 binds to Lys-Pg, the N-terminal truncated form of Pg. This interaction was firstly identified by yeast two-hybrid screen of human liver and brain cDNA libraries using the C and S domains of ADAMTS13 as the bait. This interaction was further demonstrated by the co-immunoprecipitation analysis, the far-western blotting and the Biacore system.

Under physiological conditions, Lys-Pg and Lys-plasmin are not present in circulating blood (*29*). However, in patients undergoing thrombolytic therapy using tissue plasminogen activator, low, but significant, amount of Lys-Pg was detected (*29*). Tissue plasminogen activator can be released from endothelium storage upon venous occlusion, stimulation of epinephrine or desmopressin acetate, and physical exercise. Therefore, under these conditions, Lys-Pg may be locally generated by tissue plasminogen activator and the complex of ADAMTS13 with Lys-Pg might be locally formed, thereby regulating the thrombus formation through VWF cleavage and fibrin degradation.

Physical properties of Glu-Pg and Lys-Pg are quite different. Analysis using small-angle scattering revealed that Glu-Pg has a form with the overall shape of a prolate ellipsoid by interaction between the domains in Pg (*26*). ADAMTS13 can exclusively bind to Lys-Pg but not to Glu-Pg, indicating that ADAMTS13 distinguishes the specific conformation of Lys-Pg. It is known that the conformation of plasmin is resembled to that of Lys-Pg but not Glu-Pg. It is consistent with our result that not only Lys-Pg but also Lys-plasmin can bind to ADAMTS13. Quite recently, the crystal structure of human Glu-Pg has been determined (*30*). The structure clearly showed that seven domains consisting of a Pan-apple domain, five kringle domains and a serine protease domain are loosely clustered in a diamond-shaped zig-zag assembly. Notably, the serine protease domain has a contact with kringle 2 and 4 domains. Although the structure of Lys-Pg remains to be determined, these domain contacts may differ between Glu-Pg and Lys-Pg, resulting in preferable binding of ADAMTS13 to Lys-Pg.

Recently, it was shown that ADAMTS13 is a substrate of plasmin *in vitro* (*31*, *32*). We performed a preliminary experiment as to the ADAMTS13 cleavage with plasmin (data not shown). We found that plasmin cleaved ADAMTS13 into several fragments, and the profile of those fragments was very similar to those previously reported by Crawley *et al.* (*31*) and Hiura *et al.* (*32*). Previous studies showed that the ADAMTS13 activity was progressively decreased by plasmin digestion (*31*, *32*). As for the cleavage sites, plasmin cleaved three peptide bonds, R257-A258 in the metalloprotease domain, R888-T889 in the T4 domain and R1176-R1177 in the T8 domain, but it did not cleave any peptide bonds in the C and S domains

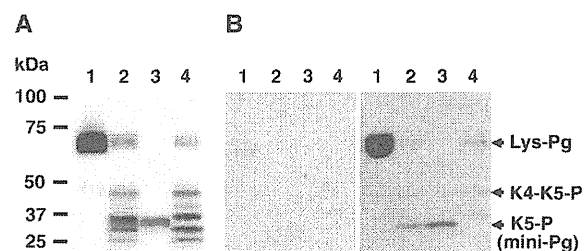

Fig. 4 Binding of ADAMTS13 to mini-Pg. Lys-Pg (lane 1, 1.28 µg protein), elastase-digested Lys-Pg (lane 2, 1.28 µg protein), and lysine-Sepharose unbound (mini-Pg, lane 3, 0.64 µg protein) and bound (several fragments containing kringle 1–4 domains, lane 4, 1.28 µg protein) fractions of elastase-digested Lys-Pg were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. (A) Coomassie Brilliant Blue staining for N-terminal sequence analysis. The N-terminal sequences of 32-kDa (lane 3) and 40-kDa (lane 4) bands were $V^{461}APPP^{465}$ and $V^{374}QDXY^{378}$, respectively, indicating that those were mini-Pg (K5-P) and a fragment consisting of the kringle 4 and 5 and protease domains (K4-K5-P), respectively. (**B**) Far-western blotting. The membrane was incubated without (left) or with (right) ADAMTS13 (4.5 µg/ml). Bound ADAMTS13 was detected by the HRP-conjugated anti-ADAMTS13 polyclonal antibody. The result is representative of three experiments.

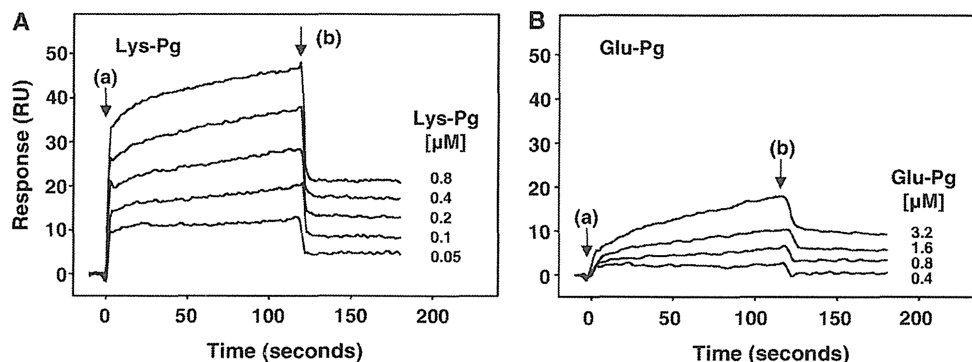

Fig. 5 Binding of Lys-Pg and Glu-Pg to immobilized ADAMTS13 using Biacore. ADAMTS13 was immobilized onto the sensor chip, and Lys-Pg (**A**: 0.05, 0.1, 0.2, 0.4 and 0.8 µM) or Glu-Pg (**B**: 0.4, 0.8, 1.6 and 3.2 µM) was injected over the ADAMTS13-immobilized sensor chip at a flow rate of 20 µl/min for 2 min. The *arrows* indicate the beginning (a) and the end (b) of the application of Pgs. Sensorgrams are shown from a typical experiment, which was repeated at least three times with similar results.

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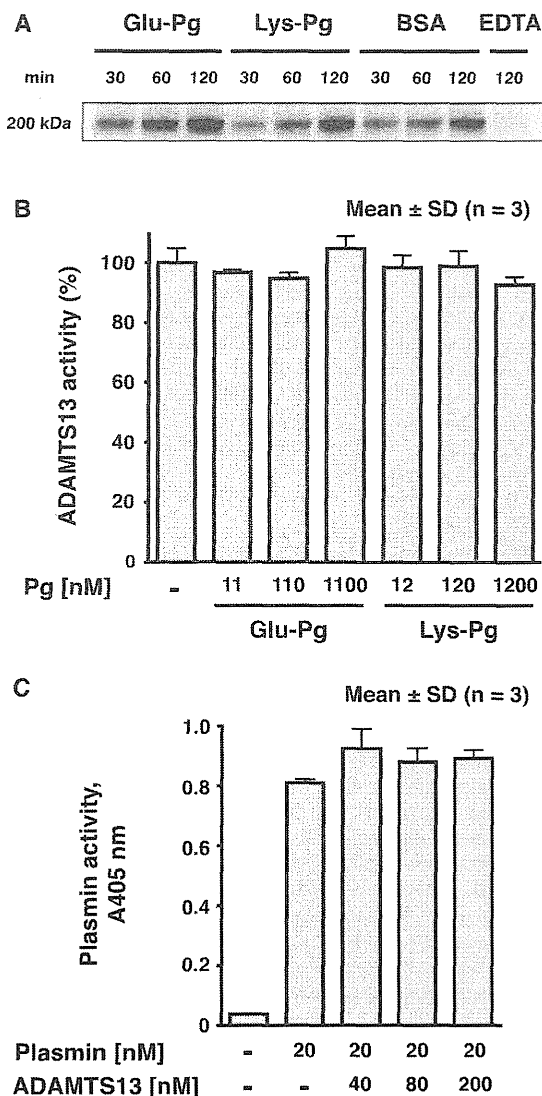

Fig. 6 Plasmin and ADAMTS13 activities in the complex. ADAMTS13 activity was assessed by the appearance of a 200-kDa fragment of VWF using western blotting (**A**) and by FRETS-VWF73 (**B**) as described under the 'Materials and Methods' section. Briefly, for VWF assay, ADAMTS13 was mixed with Glu-Pg, Lys-Pg or BSA and incubated with guanidine-pretreated VWF multimers at 37°C. The cleaved fragment was assessed by western blotting using HRP-conjugated anti-human VWF polyclonal antibody. For FRETS-VWF73 assay, ADAMTS13 was mixed with Glu-Pg or Lys-Pg and was incubated with FRETS-VWF73. Increase in fluorescence was measured with 340-nm excitation and 450-nm emission. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 min to 10 min. The relative activities were estimated from the activity of ADAMTS13 without Glu-Pg or Lys-Pg. The plasmin activity was assessed using S-2251 as substrate (C) as described under the 'Materials and Methods' section. Briefly, plasmin was preincubated with ADAMTS13 followed by the addition of S-2251. Plasmin activity was recorded as a change in absorbance at 405 nm during 30 min.

(*32*). Therefore, the fragments generated from plasmin-digested ADAMTS13 are likely to have intact C and S domains that are necessary for the plasminogen binding.

Fibrin and endothelial proteins, annexin II and α-enolase, bind to Lys-Pg through its lysine-binding site in the kringle domains (*33*, *34*). Since Lys-Pg can bind to cultured endothelial cells in a rapid and reversible fashion via the lysine-binding sites, annexin II and α-enolase are thought to be endothelial receptors for Pg. Interestingly, ADAMTS13 binds to the elastase fragment consisting of the kringle 5 and the serine protease domain of Lys-Pg. Taken together with the result of yeast two-hybrid screen, our observations suggest that the Lys-Pg binding to ADAMTS13 is a novel binding mechanism through the serine protease domain of Lys-Pg. Since the binding site of Lys-Pg to ADAMTS13 is different from that of Lys-Pg to fibrin or endothelial cells, the ADAMTS13–Lys-Pg complex might be anchored to the cells through the kringle domains of the complex. Additionally, we have demonstrated that ADAMTS13 is still active after the complex is formed. Recently, it has been shown that binding of ADAMTS13 to endothelial cells enhances its enzymatic activity (*35*).

In this study, we demonstrated ADAMTS13 binding to Lys-Pg. The physiological role of this binary complex is not clear at present; however, it might contribute to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

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Conflict of interest

K.S. is an employee of Chemo-Sero-Therapeutic Research Institute. The National Cerebral and Cardiovascular Center where T.M. and K.K. (inventors) belong has an awarded patent on the use of reagent, FRETS-VWF73. The other authors state that they have no conflict of interest.

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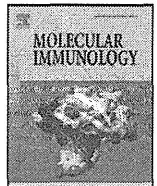

Analysis of genetic and predisposing factors in Japanese patients with atypical hemolytic uremic syndrome

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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.

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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 *CFHR*s 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).

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2.6. Mutation screening

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

2.7. Restriction fragment length polymorphism (RFLP) analysis

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

2.8. Screening for gene deletions

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

3. Results

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

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

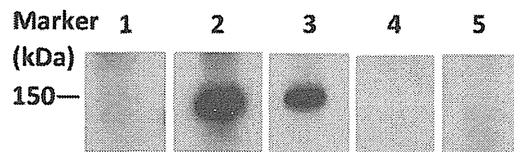

Fig. 1. Detection of CFH autoantibody in family GG. Purified CFH was electrophoresed on a 5% SDS–polyacrylamide gel and transferred to the polyvinylidene fluoride membrane. The membrane was cut into 0.5-cm wide strips and incubated with the diluted plasma sample. Horseradish peroxidase-labeled goat anti-human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

3.1. Patient X1

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

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

3.2. Patient AA1

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

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

3.3. Patient CC1

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

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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.

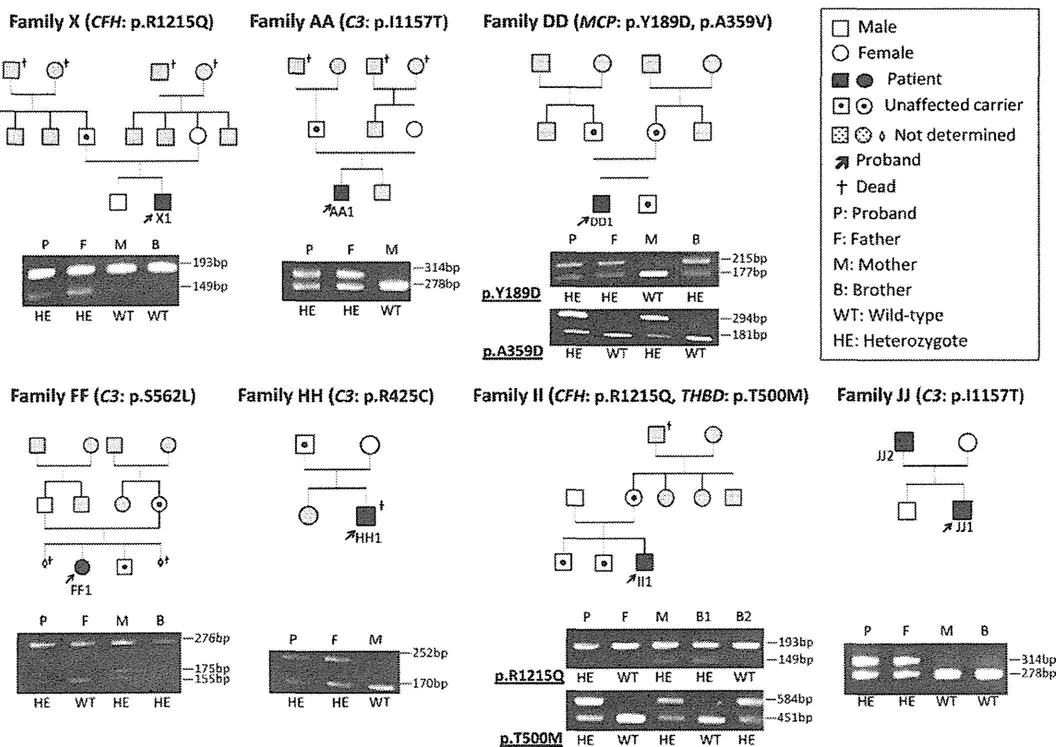

Fig. 2. Family pedigree of 8 patients with aHUS carrying causative or potentially causative mutations. Restriction fragment length polymorphism (RFLP) analyses of causative or potentially causative mutations are shown. The wild-type (WT) and heterozygote (HE) are distinguished by the electrophoretogram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

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

Patient		X1	AA1	CC1	DD1	FF1	GG1	HH1	II1	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.

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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	HE	HE	WT	WT
												II	HE	WT	HE	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	HE	HE	WT	–
												JJ	HE	HE	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).

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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

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

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

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

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

Conflict of interest

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

Contributions

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

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Appendix A. Supplementary data

Supplementary Table 1

Appendix B. Supplementary data

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

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1　未熟児網膜症姫路日赤事件最高裁判決と医療現場感覚との落差

──司法と医療の認識統合を求めて──

川 崎 富 夫

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Ⅷ まとめ

Ⅰ はじめに

訴訟では利害が対立し主張もかみ合わない。医療訴訟も同様であり，原告と被告の間はもとより，司法側と医療側，さらには医療を説明すべき鑑定医同士の間においても，考え方の隔たりは大きい。このような場における違和感は，前もって予想できる原告主張よりも，むしろ判決とその理由提示において著しい。判決に医療現場が「落差」を感じ取るのは，法と医それぞれの専門家の間で「認識の相違」があり，かみ合っていないからである。この「落差」とは，「理」にかなっていないと感じることである。判決は医療現場から乖離しており，医療側は「それでは医療が成り立たない」と受け止める。その結果「到底受け入れられない」と反発する。そもそも日本の医療は，限られた医療費の中で安全性と有効性を確保しつつ成り立つものであった。だが高度医療が望まれ，それが普及する中で，医療基盤として医療水準という概念が登場してきた。そこで，医療水準論をめぐり法学側の解釈が詳細に残されている未熟児網膜症姫路日赤事件およびその関連事件の判決をもとに，医療基盤に注目して「落差」の正体を明らかにし，法と医の「認識の統合」への道を示したい。

Ⅱ 医療水準についての理解の相違

未熟児網膜症姫路日赤事件最高裁判決[1]（以下「本件最高裁判決」という）は，法的規範としての医療水準概念を持ち出し，これに基づき網膜光凝固法を評価する。この医療水準とは「安全性と有効性」の評価の上に成り立つものとする。本件判決が医療側に伝えられ，医療上意味を持つためには「安全性と有効性」の定義が法と医で一致することが最低条件である。「安全性と有効性」についての議論は，後の本件判決の関連個所において述べることにして，ここではまず医療水準について考えてみよう。司法と医療とでは医療水準の理解が異なっている。医療の側では，新規に開始されつつある治療法

（1） 最判平成7年6月9日民集49巻6号1499頁。

の存在を前提に，医療現場で医療側が日々実際に行っている医療水準を（医）医療水準と考える。それは「現実に実施されており医療側の広い共通認識と良好な評価が得られている医療により構成される医療上の規範」と定義できる。また，そこでなされる医療を「実践的医療」と呼称する。一方，法学側が本件判決で示した医療水準を（法）医療水準と呼んでおこう。それは，「実際には広く実施されていないかまたは実施領域で共通認識が得られていないが，当該医療機関と類似の特性を備えた医療機関の状況から期待することが相当であると司法が判断する医療上の法的規範」とする。本件最高裁判決において「期待」の水準が組み込まれているが，その主語が省略された「期待」について手嶋豊教授は以下のように解説される。「患者の期待をも読み込むという立場を示したものという理解もある」が，本件差戻審判決の解釈を「(兵庫）県下での類似規模の病院間の治療体制，光凝固法の知見の普及等を基礎に，当該医療機関に期待されている内容を導き出し，ここから（姫路日赤）病院の義務違反の有無を判断しており，個々の具体的患者の期待を問題としているわけではない」と[2]。すなわち司法は社会通念を前提として「期待」を抽象的に記述する。ここでの解釈は「(患者であればいだくであろうと司法が推測し判断するところの）期待」を示すもので，それは結局「司法が思い描く期待」である。

Ⅲ　実験段階としての光凝固法

本件最高裁判決は，新規に開始されつつある治療法が「当該医療機関の類似の特性を備えた医療機関に相当程度普及している」ことをもって「期待」につながるとする。その「期待」の根拠とは，その治療の適応と手技とが一定しており，かつ「安全性と有効性」が「当該医療機関と類似の特性を備えた医療機関」において検証されていることを前提に成り立つものとする。当時光凝固法は，成人の網膜剥離治療に用いられていた。しかしここでの光凝固法は，未熟児を対象とするため眼球固定を必要とし，また見えにくい眼底を対象に，網膜上の焼灼部位も試行錯誤されていた。網膜光凝固法は侵襲的

（2） 手嶋豊「未熟児網膜症事件」別冊ジュリスト No183，142頁（2006年）。

治療であり，予想外の副作用が生じる可能性もないではなかった。本件判決において最高裁は，光凝固法について「当時において，光凝固法は有効な治療法として確立されて（いなかった)」とする原審大阪高裁の判断に対し，「診療契約に基づき医療機関に要求される医療水準についての解釈適用を誤った違法がある」と判断した[3]。だがそこで示された理由(1)～(3)については問題がある。この治療法が当時なお実験段階の治療（実験的医療）であったことを示すとともに，問題とする理由について，各(1)～(3)それぞれについて検討する。

Ⅳ　本件最高裁判決理由の(1)とそれに対する医療側の不同意

理由(1)は「光凝固法については，天理よろず相談所病院の眼科医ｆによる施術の報告後，昭和四六年ころから各地の研究者によって追試が行われ，右治療法が未熟児網膜症の進行を阻止する効果があるとの報告が相次いでいたところ，厚生省は，本症の病態や光凝固法の施術時期等に関する各地の研究者による研究成果を整理して，診断と治療に関する最大公約数的な基準を定めることを主たる目的として，昭和四九年度厚生省研究班を組織し，右研究班は，昭和五〇年三月，進行性の本症活動期病変に対して適切な時期に行われた光凝固法が治療法として有効であることが経験上認められるとし，一応の診断治療基準を示した研究成果を発表した」と明示した。そしてこれを理由に原審大阪高裁判決を違法であると結論づける。だがここには問題がある。

1　学派の存在と学派間の相違

「各地の研究者によって追試が行われ」たこと，および「右治療法（光凝固法）が未熟児網膜症の進行を阻止する効果があるとの報告が相次いでいた」ことの２点が示されたが，ここで治療の根本の「安全性」について司法が具体的に検討吟味したという形跡を示す判決文の記載はない。ここが第一の問題点である。さらに問題の上記２点について改めて筆者は，どのような

（3） 前掲注(1)。

医療機関の医師が報告していたのかを，実際に姫路日赤病院の地理的環境が近畿地方と中国地方の境に位置することを考慮して調査した。だが，その結果，学派間の研究方針の相違が明らかとなった[4]。当時光凝固法を推進する近畿地方の大規模病院は，天理よろず病院，京都大学，神戸大学，県立こども病院であり，いずれの眼科医（大学においては教授）も京都大学系列であった（県立こども病院の眼科医は，神戸大学出身であり，当時の神戸大学眼科教授が京都大学出身であることから，結局京都大学系列であるといえる)。また光凝固法に積極的な主張を行う医師についても，同様に京都大学系列の医師が大多数であった。証拠として用いられた報告は，兵庫県内の基幹病院では京大系列の出身者が多かったため，積極派（同門）の声に押され，結果的に有効との評価報告が多くなっている。これら京大系列の病院群にあっては，司法がいうように，光凝固法があたかも一つの臨床的規範（治療姿勢）をなしていたようにもうかがえる。

これに対して岡山大学およびその周辺の医療機関の眼科の当時の状況は，「岡山大学医学部では……昭和四九年三月に岐阜地方裁判所によるいわゆる日赤高山病院未熟児網膜症事件の判決を契機として，本症に関する講義も詳しくし，未熟児に対する眼底検査の指導も積極的に行うようになった。他の大学においてもほぼ右と同様の状況であった」[5]，「岡山大学医学部附属病院においても……未熟児センターが設置されたが昭和四九年までは未熟児に対する定期的な眼底検査は実施されていなかった。同病院C医師は昭和四九年一〇月本症につきはじめて光凝固による治療を試み」た[6]，また未熟児網膜症姫路日赤事件差戻審（以下「本件差戻審」という）において「岡山県下での未熟児センターを有していた病院においても，本症に取り組める眼科の専門医がいないところもあり，昭和五〇年ころの眼底検査……」[7]とあるなど，当時岡山大学および周囲の病院では，未熟児の専門的医師が光凝固法の「安全性と有効性」を低く評価してきた。そして，当時岡山大学の系列病

(4) 川﨑富夫「未熟児網膜症姫路日赤事件における医療水準の論考——医学的視点から・認識統合のために」Law & Technology 46号36頁（2010年)。

(5) 高松高判昭和58年2月24日判タ501号217頁。

(6) 高松地丸亀支判昭和61年9月17日判タ638号222頁。

(7) 大阪高判平成9年12月4日判時1637号34頁。

院では山内逸郎医師らが中心となって，対症療法としての光凝固法ではなく，網膜症の発生予防を目的とした経皮的酸素分圧測定法の実用化をまさに実現させようとしていた[8]。岡大系列の病院においては，京大系列とは異なった臨床的規範（治療姿勢）が存在するようにもうかがえる。

以上から，姫路日赤病院の周囲には，光凝固法を積極的に評価する京大学派と，そうではなく発生予防を優先し評価する岡大学派の病院が存在していた。判決は経皮的酸素分圧測定法の評価を避け，そのためその表裏関係にある光凝固法に関する岡山県の事情を過小評価することにつながった。その結果（法）医療水準の設定範囲が，京大学派の主張に偏ることになった。

2 厚生省と研究班報告

厚生省は未熟児網膜症の研究班を組織し，専門の研究者に研究を進めさせ，その研究の「安全性と有効性」の評価と，医学的および社会的意義を明らかにさせる。だが厚生省は，研究班の構成や作成した診断基準や治療基準の適否を含めて判断せず，直接関与しない立場をとる。それゆえ報告書の内容は厚生省が示す基準ではない。

本件最高裁判決および本件差戻審判決は，厚生省研究班報告まで光凝固法が各施設ごとに個々別々な診断と基準のもとに行われていたこと，そして自然治癒する群が存在することから，施術の適期についてはなお研究を要することを認め，そのことを判決文で示している。当時，統一的な診断基準を必要とした理由については，一つには自然治癒群に（無用でかえって障害を与えうる）光凝固法を実施することを避ける目的があり，また一つには岡大学派が進める経皮酸素分圧監視の効果判定に使用する目的があった[9]。そしてもう一つには，治療の適応を定めるという臨床上の大きな目的があったためである。最後の目的は，報告に羅列されているそれぞれの方法を各施設で行い，その後の結果を統一した診断基準に基づいて評価するためのものである。従ってこの研究班の治療基準とは，その後に引き続き行われる実験的医療の枠組みを，この段階でようやく決めたというに過ぎない。この報告書が診断

(8) 川﨑・前掲論文注(4)36頁。

(9) 昭和54年度厚生省研究班，未熟児網膜症の頻度に及ぼす経皮酸素分圧監視の効果，山内逸郎，厚生労働省HP，http://www.niph.go.jp/wadai/mhlw/1979/s5403048.pdf

基準を「最大公約数的」，治療基準を「平均的」と，両者を区別していること，及び治療基準が治療法を単に羅列するだけで根拠となるデータを示していないことは，単なる枠組み作成であったことのあらわれである[10]。そうするとこの治療の「安全性と有効性」とは，診断基準の統一化後に実施される症例の治療結果をもって初めて評価可能となるのである。だから，この報告時点ではまだ評価することはできない。先駆的研究者が「安全性と有効性」を個人的に確認し，それをたとえ個々系列病院内で周囲から是認されることがあっても，それで「安全性と有効性」が得られたことにはつながらない。同じ系列病院の中にあっても，研究者というものは独創性を主張し，当初別々の診断基準を持つものである。まして系列が違えば診断基準には大きな差違がある。つまり，研究班の報告の後でなければ，その「安全性と有効性」について客観的な評価はできない。厚生省研究班の診断基準が完成したのは昭和50年のことで，この研究班の報告時点では，まだ光凝固法は医学的評価がなされていない実験的医療の段階であった。

3 厚生省と医療保険制度

厚生省は医療保険制度も管轄しており，その面から「安全性と有効性」を保つ機能をもっている。姫路日赤病院，県立こども病院，天理よろず相談所病院等の保険医療機関において，患者が受ける「療養の給付」は，この医療保険制度のうえにある。「療養の給付」の具体的内容は，毎年発行される医科診療報酬点数表（社会保険研究所発行）に示されている。この内容こそが実践的医療であり，収載されたものについては「安全性と有効性」が積極的に是認されている。これに対して，医科診療報酬点数表に記載の無い診療行為は医療保険適応外であり，すなわち実験的医療である。

保険医療機関における「診療契約」とは，特別に保険外診療を前提とした契約を結ばない限り，すべて健康保険法に基づく保険診療契約である。これは，法学側が（法）医療水準を設定し解釈する上での前提条件といえる。日本の公的医療保険制度は，一疾患に対する一連の診療行為において保険診療と保険外診療（自由診療）を併用することを原則として認めない（混合診療

(10) 植村恭夫「未熟児網膜症の診断と治療」日本眼科紀要10号1286頁（1975年）。

の禁止：健康保険法は平成16年以降に関係個所の改正があり，現在は第70条，第72条，第86条が該当する）。

未熟児網膜症の網膜光凝固術が保険適応となったのは平成2年4月からである[11]。それゆえ，それまでは保険適応がない。姫路日赤病院の眼科医は保険医であり，患児は医療保険契約を結んで診療を開始した以上，当該眼科医が医療保険の療養の給付対象でない光凝固法を積極的に評価せず，またその治療を受けさせる目的で患児を県立こども病院に紹介しなかったことは，医療保険契約に基づいた正当な行為である。すなわち司法はこの判決において罰則規定をもつ医療保険制度への違反を医師と医療機関に求める判断をくだしたことになる。

あえて光凝固法を行おうとすれば，混合診療が禁止されていることから，入院診療の全てを患者（家族）が自己負担するか，あるいは病院側がすべてを負担する必要があった（極小未熟児が退院までに必要な費用は現在の金額でおよそ300～1000万円である）。実際には大学病院等で何らかの（例えば研究費等の名目で）金銭的補助がなければ実施できないものである。仮にこれを，自治体が肩代わりを行うとすれば，それは公正な医療の観点からは別の問題が生じてくる。結局当時，未熟児網膜症の光凝固法を受けさせることを目的とすれば，様々な困難が待ち受けていた。光凝固法を行うことを目的としては，保険医や保険医療機関が眼底検査を行うことも，あるいは転医させることも，さらに転医先の保険医や保険医療機関がその患児を受け入れることも，（厳密な意味において）行ってはならない行為であった[12]。厚生省は，保険外診療に関しては，その行為について「安全性や有効性」の是非を判断しない。そのことを通じて，逆に医療保険制度下の医療を「安全性と有効性」が担保されたものとして提供する。

保険外診療については，誤解が生じないよう補足が必要である。厚生省は，医療保険制度を利用しない場合にあっても，保険外診療を医師が行ってはならない診療行為とはしていない。例えば美容整形はこれに該当する。厚生省は，個々の医師の判断で実験的医療を行うことを認めており，その場合は治療に関わる検査や入院の費用全てについて，公的医療保険制度を利用しては

(11) 川崎・前掲論文注(4)36頁。
(12) 川崎・前掲論文注(4)36頁。

ならないとしている。現在でいうところの先進医療は特別なものとして除かれるが，当時の光凝固法はこれに該当しない。保険外診療は，費用負担の関係で受診者数は限られている。言い換えると，患者が「期待」に基づいて実験的医療に殺到し，多数の施術が行われることになれば，それによりもたらされるかもしれない障害の範囲を，これは限定する効果を持つ。本件姫路日赤事件において，当時の県立こども病院は保険外診療である未熟児網膜症に対する光凝固法とそのための眼底検査を行っていた。それにもかかわらず，保険外診療に期待される患者数の抑制効果が，ここでは見られない。明らかにされてはいないが，ここには保険請求上の問題（請求免除であれば違法）が絡んでいると思われる。

厚生省は，保険外診療においてその「安全性と有効性」については確認せず，すなわちその施術を良いとも悪いとも関知しない立場をとる。この厚生省の立場は，研究班の報告内容に対する姿勢と同様である。その理由は，研究班も保険外診療もどちらも「研究」領域だからである。こうして厚生省は，医療保険制度内において，被保険者が受けられる医療の「安全性と有効性」を担保する。同時に医療保険制度を利用しないことを条件に，医療の発展に必要な実験的医療への道を確保する。

4　安全性と有効性

本件最高裁判決において，司法は未熟児に対する光凝固法を「当該疾患の専門的研究者の間でその有効性と安全性が是認された新規の治療法」とする。だが，その「有効性と安全性」の定義を明らかにしていない。司法は用語使用法上「有効性と安全性」と有効性を先に記し，光凝固法の有効性の有無に主眼をおく。これに対し医学は安全性の確認に主眼を置き，これを前提にして，その後に有効性を評価する。そのため医学は実験的医療において「安全性と有効性」という語句を使用する。この時の「安全性と有効性」とは，実験的医療の研究を中止すべきと判断する重篤な有害事象が見あたらないか，又は有害事象が出現しても治療との因果関係が「否定的」であり，さらに有効性を否定できないという意味である。すなわち，「安全性と有効性」をそれぞれ二重否定できることを条件に，その研究を進めることを消極的立場で是認（消極的是認）する。言い換えると実験的医療は，「安全性と有効性」の何れかが全否定されるまで実験は続行してよいとされる。これに対し実践的医療とは，「安全性と有効性」の両者が症例によらず再現性よく揃って肯定されたものを指し，積極的に進める立場で是認されるもの（積極的是認）をいう。消極的是認の対象は結果的に医療になっていくものも，そうでないものも含むため，（医）医療水準を構成する要素とはならない。これに対して積極的是認の対象は，（医）医療水準の構成要素となる。消極的是認と積極的是認は，かつて松倉豊治教授が提案された「学問としての医学水準」と「実践としての医療水準」にそれぞれ対応する[(13)]。

未熟児網膜症についていえば昭和50年の研究班報告までは研究者毎に診断基準が異なり，そのため治療結果を研究者間で比較検討できなかった。それゆえ，この専門的研究者の是認とは消極的是認に過ぎない。そしてそのような意味での「安全性と有効性」を「期待」して患者がその治療を受けるのであれば，仮に実験的医療がうまく行かず副作用が大きいことが明らかになった場合，そしてその理由が実験的医療によるものであると後から聞かされた場合，患者の「期待」は裏切られ「欺かれた」と感じるに違いない。司法が（法）医療水準で示した「期待」とは，そのように大きく反転する「期待」である。その「期待」には，まだ医学的根拠はなく，単に実験的医療者が実験を中止する理由がないというだけの「安全性と有効性」（すなわち消極的是認）であったというに過ぎない。そのような用語が一人歩きし，また実験的医療者の説明不足が，患者と司法を混乱させたというべきである。

本件最高裁判決を導いた（法）医療水準とは，このような消極的是認に基づく「安全性と有効性」の上に成り立つ基準であった。それゆえ，一般的に理解される「安全性と有効性」とは全く無関係である。

V　本件最高裁判決理由の（2）とそれに対する医療側の不同意

理由（2）は「姫路日赤においては，昭和四八年一〇月ころから，光凝固法の存在を知っていた小児科医のc医師が中心になって，未熟児網膜症の発見と治療を意識して小児科と眼科とが連携する体制をとり，小児科医が患児の

（13）　松倉豊治『医学と法律の間』130頁（判例タイムズ社，1977年）。