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Whole-exome sequencing detects mutations in pediatric patients with atypical hemolytic uremic syndrome in Taiwan

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ABSTRACT

Although atypical hemolytic uremic syndrome (aHUS) is a genetic disorder, molecular defects are detected in only 60% of patients. We aim to dissect the genetic background by whole exome sequence and the clinical characteristics of pediatric patients with aHUS. Ten patients (6 male and 4 female) with mean age 5.2 ± 5.0 years were enrolled. The age at onset ranged from 2 days to 11 years. Eighteen different mutations (17 missense, 2 nonsense, and 11 novel) on 7 complement and 3 coagulation genes were detected in all patients. The majority of mutation was heterozygous and S1191L on *CFH* were the recurrent mutation. Sixty percent of patients had multiple genetic mutations. Nine mutations were associated with genes known to be implicated in aHUS (*CFH, CFI, CD46, CFHR5, and DGKE*), while 4 and 5 mutations were detected on complement- (*C8B, C9, and MASP1*) and coagulation-associated (*VWF* and *CD36*) genes, respectively. *CD36* may be a candidate gene act as disease modifier for aHUS through the contribution of thrombosis by impairing the interaction with TSP-1 and ADAMTS 13 shown in simulation model. Genetic defects on both complement and coagulation pathways play pathogenic roles on aHUS. *CD36* may be a novel candidate gene act as disease modifier of aHUS.

1. Introduction

Atypical hemolytic uremic syndrome (aHUS) is a life-threatening disease featuring thrombotic microangiopathy, characterized by complement dysregulation and aberrant activation of alternative complement pathway [1]. Loss-of function or gain-of-function on genes encoding complement and coagulatory proteins have been recognized as key to etiopathogenesis of this type of thrombotic microangiopathy. Specifically, uncontrolled generation of membrane attack complex leads to endothelial damage of vital organs including kidney, heart, and central nervous system [2–5]. In the past decades, genetic defects involving the regulation of complement and coagulation activations have been demonstrated to be the cause of aHUS [2,4]. Although aHUS is primarily a genetic disorder, only up to 60% patients harbored abovementioned genetic abnormalities [3,4]. The limited detection rate may be secondary to method of testing (ie. direct target gene sequencing), which leads one to believe that other gene mutations that contribute to this disease process, remain undiscovered. Furthermore, recent studies have described incomplete penetrance, racial difference and heterogeneous clinical characteristics of aHUS [1,4,6]. The finding of novel

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mutations or variants may further clarify the relationship between genotype and phenotype.

So far, comprehensive analysis of genetic background of pediatric patients, especially in Asia, is limited. In addition, follow-up outcome of pediatric patients with aHUS are not well studied. Our aim for this study is to investigate the clinical, laboratory manifestations, genetic characteristics, and follow-up outcome in pediatric patients with aHUS.

2. Materials and methods

2.1. Subjects and diagnosis

The study protocol was approved by the Ethics Committee on Human Studies at Chang Gung Memorial Hospital, in Taiwan, R.O.C. (IRB 0201701388A3). Informed consent was obtained from the patients and their parents after a detailed description of the study. Between 2010 and 2018, ten pediatric patients with aHUS were enrolled. Thrombotic microangiopathy is defined as thrombocytopenia and microangiopathic hemolytic anemia. aHUS is defined as thrombotic microangiopathy with ADAMTS activity > 10%. Patients with known coexisting diseases that could contribute to the diagnosis of hemolytic uremic syndrome were excluded [7–9].

2.2. Molecular analysis by whole exome sequence (WES) and Sanger sequencing

Genomic DNA was isolated from peripheral venous blood sample. The genes involved complement and coagulation pathways were analyzed. Direct Sanger sequencing was performed for all patients and their parents to verify the genetic variants detected by whole exome sequencing. We performed exome capture using the Agilent SureSelect Human All Exon Kit 58 m (v6) (Agilent Technologies) and massively parallel sequencing using the HiSeq 4000 platform (Illumina, San Diego, CA) to generate paired-end 150-bp reads from genomic DNA sequencing in Biotools (New Taipei city, Taiwan). Raw image analyses and base calling were performed using Illumina's Pipeline with default parameters. Sequence data were aligned to the reference human genome (hg19) using the Burrows-Wheeler Aligner (BWA), and duplicate reads were removed using Picard tools [10]. We used the Genome Analysis ToolKit (GATK) to perform the re-alignment and variation (SNP and InDel) detection [11]. Annovar was utilized to catalogue the detected variations [12]. Then, we filtered variations with a homopolymer length > 6 (and synonymous substitutions) or that were common (> 2%) in dbSNP150 (http://www.ncbi.nlm. nih.gov/projects/SNP/), HapMap, the 1000 Genomes Project (http://www.1000 genomes.org), the Exome Aggregation Consortium (ExAC) database and the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org). Integrated genome viewer (IGV) was used to visualize the reads and comparing polymorphisms between each sequenced individuals. Pathogenicity score was calculated using SIFT, PolyPhen2, LRT, MutationTaster, FATHMM, M-CAP, CADD, and GERP [13–20]. Pathogenicity score of 3 was considered as pathogenic. All variants were excluded the single neucleotide polymorphism by analyzing in 100 health subjects. Recurrent mutation was defined as the reappearance of the same mutation in at least two unrelated patients.

2.3. Phenotype analysis

Demographics, initial clinical manifestations, laboratory data, treatment, and follow-up outcome were collected prospectively in all patients. Clinical features, such as gender, age at onset, family history, underlying disease, blood pressure, symptoms at presentation, trigger events, time from trigger to disease onset, and organ involvement were recorded. Laboratory exams including hemoglobin, platelet count, schistocyte on smear, coombs test, creatinine, lactate dehydrogenase, amylase, lipase, ADAMTS 13 activity, complement 3 (C3), complement 4 (C4), CH50, vitamin B12, homocysteine, antinuclear antibody, antiphospholipid antibody, urine analysis and streptococcus pneumonia antigen, and stool for shigatoxin-producing *E. coli*. were collected on initial presentation. Treatment response to plasma infusion, plasma exchange, and eculizumab (see below for definition) and follow-up outcomes including sequelae of vital organs were recorded.

2.4. Determination of serum complement levels

Blood obtained before plasma therapy were used for determination of serum complement levels. The levels of serum complement factor H (CFH) (Abnova), complement factor I (CFI) (LSBio), and C5b-9 (Blue Gene Biotech) were measured using ELISA according to the manufacturer's guidelines. The linear portion of the standard curve was subsequently used for the measurement of serum CFH, CFI, and C5b-9. All assays were run in duplicate and when standard errors were over 10%, samples were routinely re-analyzed.

2.5. Determination of anti-CFH autoantibody

For determination of anti-CFH autoantibody, the blood draw before plasma therapy was used for detection. The presence of autoantibody against CFH was determined by using CFH IgG ELISA kits (Abnova, Taipei, Taiwan) according to the manufacturer's instructions.

2.6. Structure models of CD36 mutant and interaction with ADAMTS13

The resolved structure of human CD36 (PDB code: 5LGD) was used as a template to generate the E177V/R273G and R386W mutants using the Built Mutants protocol (Biovia Discovery Studio 2017). The geometries of the models were optimized using the algorithm of smart minimization in CHARMm force field including the implicit solvent model of generalized born in the calculation.

The ADAMTS13-MDTCS model (including metalloproteinase (M), disinterring-like (D), TSR (T1), cysteine-rich (C) and spacer (S) domains) was build using the Homology Modeling protocol (Biovia Discovery Studio 2017) with the crystal structures of ADAMTS13-DTCS (PDB code: 3GHN) and ADAMTS4 (PDB code: 2RJP) as template [21,22]. The best model was submitted for energy minimization using the CHARM as the applied force field. Docking of wild type and E177V/R273G mutant CD36 (PDB code: 5LGD) with ADAMTS13-MDTCS model employed the ZDOCK protocol which set as the receptor and ligand proteins, respectively. The scoring function of ZRank was used to score the docked protein poses. The docked protein poses were then refined using the protocol of RDOCK.

2.7. Treatment and follow-up outcome

All patients underwent plasma therapy (plasma exchange or plasma infusion) according to published guideline [23]. Response to plasma therapy is defined as the normalization of platelet count and hemoglobin level, serum LDH, and at least 25% reduction of serum creatinine after 5 daily plasma therapies [23]. Response to eculizumab is defined as transfusion independence with normal platelet count and hemoglobin level, absence of new-onset TMA, and serum LDH < 1.5 times the upper limit of normal in ≥2 consecutive measurements for ≥4 weeks [24]. Complications and side effects of plasma therapy and eculizumab were analyzed, respectively. The follow-up outcomes of survivals were also recorded.

2.8. Statistical analyses

To determine the correlation between genotypes and phenotypes, Fisher's exact test was used to compare their differences. All the *P* values were adjusted by sex for linear regression, and P 0.05 was considered statistically significant.

Table 1
Demographic and clinical characteristics at presentation.

Patient	1	2	3	4	5	6	7	8	9	10
Gender	Male	Female	Male	Male	Male	Male	Male	Female	Female	Female
Onset age	4Y	2D	11Y	4Y	4Y	3Y	1.5Y	14Y	3M	2Y
Symptoms	Bloody diarrhea Oliguria	SOB[a] Oliguria	Abdomen pain Vomiting	Jaundice	SOB[a]	Oliguria Headache	Bloody diarrhea	Oliguria	Bloody diarrhea Oliguria	Bloody diarrhea Oliguria
Trigger event	RTI[b] (Enterovirus)	No	No	RTI[b] (Enterovirus)	RTI[b] (Enterovirus)	No	GTI[c]	No	RTI[b]	GIT[c]
Creatinine (mg/dl)	6.5	1.3	7.8	1.6	14	1.8	2.5	7.4	4.6	4.1
Hemoglobin, g/dl	9.4	10.6	6.9	6.2	5.5	7.0	8.2	8.3	7.4	8.0
Platelet, 1000/μl	118	76	30	46	99	8	32	69	40	19
Lipase/Amylase, U/L	60/15	55/20	65/25	78/26	380/440	70/15	100/25	70/20	56/18	40/24
C3/C4[d], mg/dL	55/10	34/2	90/20	114/32	68/17	84/21	75/18	90/24	68/8	78/12
C5b-9[e], ng/mL	320	[f]	[f]	[f]	760	266	462	340	422	280
CFH[g], mg/L	336	[f]	[f]	[f]	221	527	364	186	368	348
CFI[h], mg/L	48	[f]	[f]	[f]	1.3	42	7.1	70	46	62
Extra-renal Involvement	Brain Heart GIT[c]	Lung	–	Brain Heart	Brain Heart Pancreas	Heart	GIT$^+$	–	Heart[g] GIT$^+$	GIT$^+$

[a] Shortness of breath.
[b] Respiratory tract infection.
[c] Gastrointestinal tract infection.
[d] Normal range 90–120/10–20.
[e] Normal range 127–400.
[f] Not perform.
[g] Normal range 330–680 mg/L.
[h] normal range 40–80 mg/L.

3. Results

3.1. Demographic and clinical manifestations

Ten pediatric aHUS patients (6 males) of mean age 5.27 ± 5.0 years (ranging from 2 days to 14 years) were studied. Over one-third (4/10) of patients had disease onset at < 2 years of age. None of the patients had familial history of aHUS or underlying disease. As shown in Table 1, oliguria, bloody diarrhea, shortness of breathing, and abdominal pain, were the most common manifestations at presentation. Hypertension was present in all patients. Infectious triggers were identified in 6 patients (60%), and the interval from triggers to onsets ranged from 2 to 10 days. All patients had acute kidney injury at presentation and four of them had histological evidence of renal thrombotic microangiopathy. The heart, brain and gastrointestinal tract were the most commonly involved extra-renal organs. Five patients had heart failure and three had seizures at presentation. Gastrointestinal involvement presenting as bloody diarrhea and pancreatitis were noted in 4 and 2 cases, respectively. Of note, we excluded the shigatoxin-producing *E. coli*. and other infectious pathogens by special cultures and viral isolations in aHUS patients with bloody diarrhea.

3.2. Mutations of corresponding genes

We analyzed the genetic variants involved complement and coagulation pathways by WES. Sanger sequencing was used to verify the variants from patients and also their parents. Whole exome sequencing followed by validation of Sanger sequencing were conducted in all 10 patients, and the quality of sequencing, variant position, transcript change, and pathogenicity score were shown in supplement Table 1 and Table 2. Ten patients had reported or novel nonsynonymous mutations on 10 genes including *CFH*, *CFI*, *CFHR5*, *CD46, CD36*, *C8B, C9*, *MASP1*, *VWF*, and *DGKE*. Complement genes reported to be associated with aHUS included *CFH*, *CFI*, *CFHR5*, *CD46*, *C8B, C9*, and *MASP1*. Coagulation-associated genes reported to be associated with aHUS were *VWF*, *DGKE*, and CD36. Of note, *CD36* was the novel gene and has not been reported to be associated with aHUS. To evaluate the possible pathogenicity of these variants, we used computational prediction methods. Eighteen different mutations, 11 novel variants, were predicted to be deleterious. Among these 18 different mutations, 17 and 2 are missense and nonsense, respectively. Ten of 18 mutations (56%) were associated with genes known implicated in aHUS (*CFH*, *CFI*, *CD46*, CFHR5, and *DGKE*.). Four mutations were from other genes involving complement system (*C8B*, *MASP1*, and *C9*), and 5 were from gene associated with coagulation system (*VWF* and *CD36*) (Fig. 1). The majority of the mutations detected in this cohort of aHUS were heterozygous. S1191L is the recurrent mutation of *CFH*. Three patients (30%) had combined defects on complement genes and/or coagulation genes, and another 3 patients harbored multiple defects on different complement genes.

3.3. Laboratory characteristics

All patients manifested the triad of thrombotic microangiopathy (anemia, thrombocytopenia and acute kidney injury). Overall, 9 patients (90%) had severe anemia (Hgb level < 10 g/dL) and 90% had severe thrombocytopenia (PLT < 100,000/uL). All patients had schistocyte on smear, proteinuria and urine occult blood. Elevated serum amylase and lipase levels were found in 2 patients. All had normal ADAMTS 13 activity (range from 55 to 94%) with absence of anti-nuclear and anti-phospholipid antibody, urine pneumococcus antigen, and shigatoxin-producing *E. coli* (STEC) on stool. All patients had serum vitamin B12 level within the normal range.

3.4. Serum complement and anti-CFH autoantibody studies

As shown in Table 1, three patients (30%) had normal plasma C3 levels. Seven patients had low C3 levels, and two of them also had low C4 levels. Seven patients received complete complement studies. At onset of aHUS, high serum CH50 levels in 7, elevated plasma C5b-9 levels in 3, low CFH in 2, and low CFI levels in 2 patients in whom levels were determined. Serum anti-CFH antibody was analyzed in 7 patients, of which all were negative.

Table 2
Genetic defects.

Patient	Gene	Nucleotide	AA change	Related disease	PS[b]	gnomAD all[c]	gnomAD EAS[c]	1000 EAS[d]	EXAC EAS[e]	dbSNP150	Inheritance
1	*CFH*	c.T3172C	Y1058H[a]	aHUS	3	0.001	0.008	0.005	0.010	rs55679475	Maternal
	CFH	c.G3178C	V1060L	aHUS	5	0.001	0.007	ND	0.010	rs55771831	Maternal
	CD46	c. C293T	T98I[a]	aHUS	3	1.78E-04	0.002	0.005	0.003	rs116800126	Paternal
2	*CFH*	c. C3572T	S1191L	aHUS	4	0	0	ND	ND	Novel	Paternal
	CD36	c.C1451T	R386W	–	8	0.001	0.003	0.003	0.002	rs148910227	Paternal
	CD36	c.C1704T	T470I[a]	–	3	9.63E-05	0.001	0.002	0	ND	Maternal
3	*CFH*	c. C3572T	S1191L	aHUS	4	0	0	ND	ND	Novel	Maternal
	CD46	c. A86C	Y29S[a]	aHUS	3	1.59E-05	2.18E-04	ND	1.00E-04	rs756273271	Maternal
4	*MASP1*	c.G2035A	G679R[a]	Immunodificiency	8	3.86E-04	0.005	0.004	0.005	rs3774266	De novo
5	*CFI*	c.G904 T	E302X[a]	aHUS	–	0	0	ND	ND	Novel	Maternal
6	*DKGE*	c.C390G	D103E[c/a]	aHUS	7	3.19E-05	3.81E-04	ND	3.00E-04	rs757482556	Paternal
7	*CFH*	c. C3572T	S1191L	aHUS	4			ND	ND	Novel	Paternal
	VWF	c.C4499A	A1500E	VWD	5	2.59E-04	0.004	0.002	0.002	rs61750096	Maternal
8	*C9*	c.C346T	R116X	C9 deficiency	–	0.001	0.010	0.014	0.011	rs121909592	De novo
9	*CFH*	c.G2509A	V837I	aHUS	3	0.001	0.018	0.016	0.017	rs55807605	Maternal
	CFHR5	c. G700A	E234K[a]	aHUS	6	7.79E-05	0.001	ND	0.001	rs755972876	Paternal
	MASP1	c.C379T	R127C	Immunodificiency	7	4.96E-05	0.001	ND	0.001	rs115647447	Paternal
10	*C8B*	c. A352T	S118C[a]	aHUS	6	0.001	0	ND	ND	rs199745832	Maternal
	CD36	c. A825T	E177V[a]	–	7	0	0	ND	ND	Novel	Maternal
	CD36	c. A1112G	R273G[a]	–	8	1.06E-05	0	ND	1.00E-04	rs778804205	Paternal

AA mean Amino acid; ND, no data; *CFH*, complement factor H; *CFHR5*, complement factor H related 5; *CFI*, complement factor I; *CD46*, membrane cofactor protein 46; *DGKE*, diacylglycerol kinase epsilon; *C8A*, complement component 8 alpha polypeptide; *C9*, complement component 9; *MASP1*, mannan binding lectin serine peptidase 1; *VWF*, Von Willebrand factor; *CD36*, membrane cofactor protein 36.

[a] Novel.

[b] PS, pathogenicity score was calculated using SIFT, PolyPhen2, LRT, MutationTaster, FATHMM, M-CAP, CADD and GERP, Novel variants are those variants not reported in the gnomAD All, gnomAD EAS, the EXAC EAS, 1000 Genomes, or dbSNP databases.

[c] Genome Aggregation Database (gnomAD) ALL population and East Asian.

[d] 1000 g EAS, 1000 genome project East Asian.

[e] ExAC, The Exome Aggregation Consortium.

3.5. Simulation model of CD36 wild-type and mutants

The resolved structure of human CD36 (PDB code: 5LGD) covering amino acid residues from 35 to 439 was used as our analyzing template. Hence, the CD36 mutation (R386W/T470I) of patient 2 only was analyzed the effect of R386W in our current simulation model (Fig. 2A–D). We further analyzed the protein stability free energy difference of folding between mutant and the wild type proteins and calculated the mutation energy values of these two CD36 mutation: 5.9 kcal/mol for R386W and 7.3 kcal/mol for E177V/R273G, respectively. Both values were over 0.5 kcal/mol suggests that two mutant proteins are less stable than wild-type CD36 protein. The important structure differences of two CD36 mutants (R386W and E177V/R273G) were described below (Fig. 1A–D). The hydrogen bond interactions from the side chain of R386 with the NAG were decreased after the mutation to W386. The mutation destabilized the binding affinity in the protein-ligand complex (~1.6 kcal/mol). The hydrogen bond and salt bridge interaction of E177 with R173 were lost after mutated to V177. The hydrogen bonding and hydrophobic interactions from the side chain of R273 with S274, A299, W239, I271, F312, and L328 stabilized the conformation of the protein that was lost after mutation to G273.

Sequences of 93–110 and 139–155 in CD36 were reported as TSP-1 binding sites [25]. Our analysis demonstrated that the interaction sites that are missing at the interfaces of the CD36 (E177V/R273G) mutant and ADAMTS13 are shown as black circles in (Fig. 1E). The interaction sites included the salt bridge between His657 and Glu515 and hydrogen bonding between Arg183 and Val424 from wild-type CD36 and ADAMTS13, respectively.

3.6. Treatment and outcome

Seven patients (70%), as shown in Table 3, needed renal

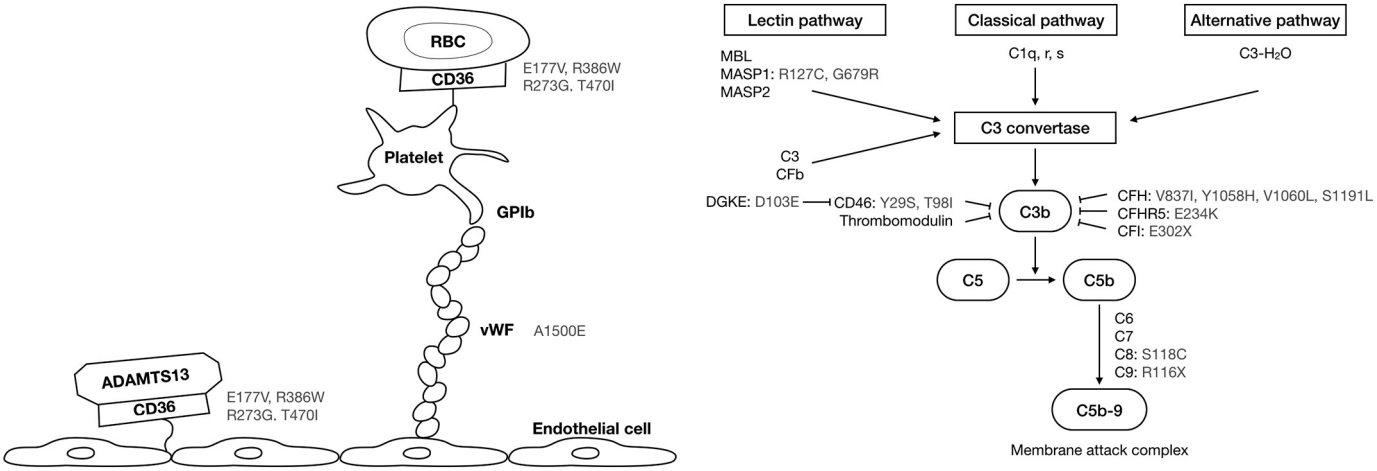

Fig. 1. Illustration of mutations on complement pathway and coagulation cascade.

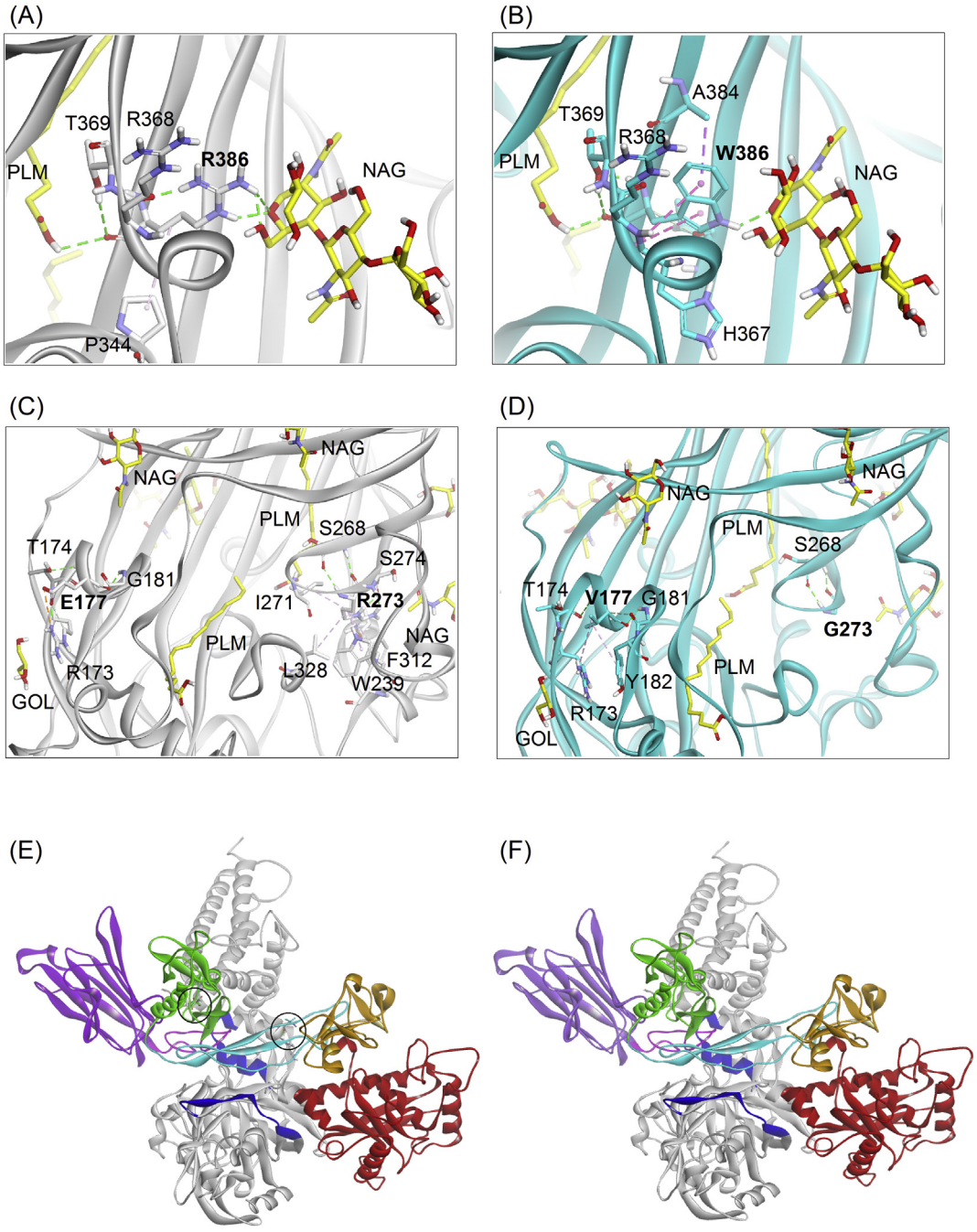

Fig. 2. The models of human variant CD36. The proteins were present as ribbon model and colored as white and cyan for wild-type (A: R386 and C:E177/R273) and mutant CD36 (B:W386 and D:V177/G273), respectively. The residues involved in interactions are shown as stick model. The ligands of palmitic acid (PLM), *N*-acetyl-D-glucosamine (NAG) and glycerol (GOL) were presented as yellow for the carbon atom. The hydrogen bond, electrostatic and hydrophobic interactions were shown as dashed green, orange and pink lines, respectively. Wild type (E) and E177V/R273G mutant (F) CD36 are shown as white color. Sequences of 93–110 and 139–155 in CD36 are presented as blue color. $MDTC_{\alpha}C_{\beta}S$ domains in ADAMTS13 are colored as red, bright orange, cyan, green, magenta and violet, respectively. The interaction sites that are missing at the interfaces of V177/G273 mutant CD36 and ADAMTS13 are shown as black circles in (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

replacement therapy due to acute kidney injury. Nine patients were treated with plasma therapy, 7 were initiated within 24 h and 2 within 48 h from onset of disease. Three of nine patients (33.3%) responded to plasma therapy. Of note, three patients (3/9, 33.3%) developed catheter-related bacteremia during plasma therapy. Patient 9 who harbored combined mutations on *CFH*, *CFHR5*, and *MASP1* genes presented with fulminant heart failure with acute kidney injury and died before initiation of plasma therapy. Patient 2 received plasma infusion due to small body size and unavailability of extracorporeal equipment. Five patients received eculizumab as second line of treatment due to either poor treatment response to plasma therapy (4 patients) or drug availability (1 patient) after first episode of aHUS, and all of them responded to eculizumab. Two patients, patients 2 and 9, died of pulmonary hemorrhage and heart failure during their first episode of aHUS. Eight patients were followed for a mean of 5.5 years (ranging from 6 months to 12 years). There were no opportunistic infection or other side effects in patients on continuous eculizumab therapy. Patient 1 was initiated eculizumab therapy during relapse of thrombotic

Table 3
Treatment and outcome.

Patient	1	2	3	4	5	6	7	8	9	10
Renal replacement therapy	CVVH[a]	No	No	HD[b]	CVVH[a]	No	CVVH[a]	CVVH[a]	CVVH[a]	HD[b]
Plasma therapy (PI/PE)[c]	Yes (PE)	Yes (PE)	Yes (PE)	Yes (PE)	Yes (PE)	Yes (PE)	Yes (PE)	Yes (PE)	–[e]	Yes (PE)
Response	Yes	No	No	No	No	No	Yes	No		Yes
Eculizumab (first/second line)	No[d]	Yes (second)	No	No	Yes (second)	Yes (second)	Yes (second)	Yes (second)	No	No
Response		Response			Response	Response	Response	Response		
Outcome after first episode										
Mortality	No	Yes	No	No	No	No	No	No	Yes	No
Cause		Pulmonary hemorrhage							Heart failure	
Follow-up outcome										
Duration of follow-up	5.5 years		3 years	11 years	1 year	6 months	1 year	2 years		6 months
Relapse of TMA	Yes		No	No	No	No	No	No		No
CKD[e] (stage)	Yes (V)		Yes (III)	Yes (V)	Yes (IV)	No	No	Yes (V)		No
Extra-renal sequels[-f]	Heart failure		No	Heart failure	Cerebral infarction Heart failure	No	No	No		No
Mortality (causes)	No		No	No	Yes (Sepsis)	Yes (Heart failure)	No	No		No

[a] Continuous venovenous hemofiltration.
[b] Hemodialysis.
[c] Plasma infusion/plasma exchange.
[d] Pre-eculizumab Era.
[e] Not perform due to sudden death
[-f] Chronic kidney disease.

microangiopathy. There were no relapses found in patents on continuous eculizumab treatment. End-organ injury that were noted at follow-up included: chronic kidney disease (5), heart failure (3), and cerebral infarction (1), respectively. During follow-up, patient 5 and 6 died of sepsis and heart failure, respectively.

3.7. Correlation between phenotype and genotype

aHUS patients were divided into single genetic defect and multiple genetic defects, with and without combined defects on complement and coagulation genes for analyzing correlation between genotype and phenotype. No statistical correlations were found between genotypes above-mentioned and organs involved, therapeutic response, and disease outcome, which include chronic kidney disease and mortality at follow-up (Supplement Table 2).

4. Discussions

We report a nationwide pediatric aHUS study in Taiwan focusing on the phenotypic characteristics, genotype, and long-term follow-up outcome. Over one-third of patients with aHUS are < 2 years of age at onset and extra-renal involvement such as bloody diarrhea, and normocomplementemia are not uncommon. Eighteen different mutations including reported or novel non-synonymous defects were detected from 10 different genes on both complement and coagulation genes in all patients by WES. Nine variants were from genes known to be involved in complement and coagulation pathways. Of note, *CD36* not been reported may act as a modifier gene of aHUS. Specifically, S1191 L was the recurrent mutation on the *CFH* gene. There were no significant differences on follow-up mortality, renal, and extra-renal sequelae for those with or without combined mutations. Heart failure and chronic kidney disease are the most common sequelae during follow-up.

Over one-third of our pediatric patients are < 2 years, which is in line with previous studies [26–28]. Recent studies indicate that a trigger is required for onset of disease. In our cohort, we found 60% to have trigger events, of which all were infections [2,29]. This rate is less than around 80% reported in younger children, but higher than that reported in adult aHUS patients [7,23,[30]]. Eighty percent of patients had extra-renal manifestations at presentation in this study, which is higher than 20–30% reported by previous studies [31,32]. Patient 9 was highly suspected to have cardiac thrombotic microangiopathy by the presence of elevated cardiac enzyme and sudden death. Although heart failure was the most common extrarenal involvement, it is difficult to differentiate between direct involvement due to cardiac thrombotic microangiopathy and heart failure secondary to severe hypertension. Previous reports have shown that about 3% of patients have cardiac microangiopathy, which can contribute to sudden death [2,33]. Bloody diarrhea, the cardinal feature of HUS caused by shigatoxin-producing *E. coli* (STEC-HUS), is not uncommon in our patients (40%). This rate is higher than a previous study in Japan and can easily be confused with STEC-HUS [34]. The shigatoxin-producing *E. coli* and other possible pathogen responsible for bloody diarrhea were excluded by several cultures and viral isolations in current study, therefore, the bloody diarrhea may possibly result from the bowel thrombotic microangiopathy. In summary, pediatric aHUS patients tend to have more identifiable triggering events, extra-renal manifestations, and bloody diarrhea.

Until now, there is no definitive test for the diagnosis of aHUS. Although aberrant activation of alternative complement pathway plays an important role in pathogenesis of aHUS, decreased plasma C3 level was found in 60% of our patients and prior studies [2,4,35]. This illustrates that normal plasma C3 level does not exclude the diagnosis of aHUS. Only one patient with *CFH* mutations exhibited decreased plasma CFH level and one patient with *CFH* mutation had low plasma level of CFI in our study. These findings are in accordance with the observations of prior studies, which showed that normal plasma CFH and CFI levels do not rule out the possibilities of defects of corresponding genes [4,8]. C5b-9, the terminal products of complement activation, has been considered to be the marker of overactive complement activation [36]. However, our current finding and previous studies both showed that only half of aHUS have increased serum C5b-9 during the acute phase of the disease [37]. To explain this finding, two speculations were considered. One is that the timing of sampling at acute onset may not have corresponded with the peak levels of plasma

C5b-9, due to its relative short half-live. The other is that C5b-9 was generated and deposited at the sites of damaged tissue during complement overactivation and hence was not always detected in elevated levels in the serum.

Up to 60% of patients with aHUS were found to have mutations on *CFH*, *CFI*, *CD46*, *C3*, *CFB,* or *DGKE* and previous studies demonstrated incomplete penetrance in familial aHUS [38–40]. These findings indicate that other genetic contributors are necessary for disease [2,31,32]. By using WES, all of our aHUS cohorts were found to harbor defects on complement and/or coagulation genes. These detected defects are deleterious nonsynonymous variants either involved in complement genes or genes that play critical roles in coagulation. These variants are pathogenic based on the structural changes demonstrated by simulation models and significant pathogenicity score predicted by computational prediction methods. Two *MASP1* variants, one *C9* variant, and one *C8B* variant were identified from 4 aHUS patients in this study, and these variants have been identified in cases of aHUS [42]. *MASP1* is a gene involving lectin complement pathway, and *C8B* and *C9* are corresponding genes for members of complement attack complex. Although the true impact of these variants on development of thrombotic microangiopathy remain unknown, Patient 8 with genetic defect on *C9* achieved remission of thrombotic microangiopathy after eculizumab treatment, which may suggest that the underlying thrombotic microangiopathy could be a result of a broad range of complement abnormalities. Our study showed that combined mutations on complement and coagulation-associated genes were found in 30% of patients, which is higher than that of prior studies (3–12%) [26,34,40]. Our findings are consistent with prior findings that both complement and coagulation pathways are associated with aHUS [41]. Patient 7 harbored combined defects on *CFH* and *VWF* genes. Von Willebrand factor (vWF) is a large glycoprotein and play an important role on thrombosis and homeostasis. The alteration of coagulation process by the genetic variant on vWF may lead to the formation of thrombi in small vessels, mechanical destruction of red blood cells, and ultimately, thrombotic microangiopathy. Therefore, This *VWF* variant detected in this patient with *CFH* variants was speculated to be the disease modifier. Further studies with a larger sample size or a gene registry for aHUS are warranted to confirm our findings.

Two of our aHUS patients with mutations on complement genes also harbor defects on an unpublished gene, *CD36*. CD36 is a membrane glycoprotein with receptor of thrombospondin, and is mainly expressed on an extensive range of cells and tissues, including microvascular endothelial cells, platelet, macrophages, monocytes, adipocytes, breast, and gut. CD36 interacts with fatty acid, modified LDL, thrombospondin-1 (TSP-1), and Toll-like receptor to play physiological roles on lipid metabolism, coagulation and platelet function, and innate immunity, respectively. The interaction of CD36 on platelet with TSP-1 on endothelial cells has been shown to involve the platelet activation and thrombus stabilization [43]. Prior study demonstrated that the presence of serum antibody directed against CD36 could result in vascular damage and thrombosis formation in lupus patients with thrombotic complication [44,45]. Therefore, the abolishment of CD36 may lead to thrombosis. Recently, there is a speculation that endothelial CD36 may play a role in localizing and forming a reservoir of ADAMTS13 on microvascular endothelium [46]. In agreement with previous findings, our simulation model demonstrated the *CD36* mutations found from our patients would destabilize the structure of CD36 protein, which may further contribute the formation of thrombotic event. Although the missing interaction sites included the salt bridge between His657 and Glu515 and hydrogen bonding between Arg183 and Val424 from wild-type CD36 and ADAMTS13, the destabilized CD36 itself may diminish the consequent function of binding between CD36 on endothelial and TSP1on ADAMTS13. Therefore, the development of TMA in cases with *CD36* mutation may result from the impairment of activation of platelet and coagulation caused by diminishment of endothelial TSP-1expression and/or endothelial localization of ADAMTS13. The exact consequence of ADAMTS13 or TSP-1 binding to endothelial CD36 remains to be further verified. Although both of these two patients with CD36 mutations reached to remission after eculizumab therapy, the exact role of CD36 in the pathogenesis in aHUS patients with genetic defects on complement requires further clarification.

Plasma therapy provides functional complement regulatory proteins, however, only 30% of patients with plasma therapy reached remission. This response rate is less than the reported 50% in Italian cases [2]. Five patients received eculizumab for second-line therapy during the first episode of aHUS due to non-response of plasma therapy, and all of them reached remission of thrombotic microangiopathy without relapse during follow-up. However, three of them developed CKD during follow-up. These patients had higher mean serum creatinine levels and blood pressure at presentation than those without CKD. In line with our results, Jamme et al. identified that the magnitude of renal dysfunction, high blood pressure, and higher platelet count at time of diagnosis were predictors of CKD in aHUS [47]. In addition, other factors including delayed treatment, poor response of plasma therapy in pre-anti-C5 Era, or disease severity may also contribute to the unfavorable renal outcome. Importantly, there were no differences in organs involved, response of treatment, and outcomes between patients with single genetic defects and multiple genetic defects in this study. Furthermore, we also did not find significant differences in mortality and renal outcomes between patients with and without combined complement and coagulation mutations the different gene mutations. The genotype-phenotype correlation remains inconclusive in this study, which is at least partially due to the small sample size or other unidentified genetic defects associated with phenotype.

The current study has some limitations. First, a small patient size limits our ability to determine the correlation between phenotype and genotype. Further studies to confirm this interpretation of our results are required. Second, we were unable to collect a complete data set for serum complement regulatory proteins levels.

5. Conclusions

In conclusion, defects on genes involved in complement and coagulation pathway may contribute to the development of aHUS. CD36 may be a novel candidate gene for a disease modifier. No correlation between genotype and phenotype as well as no difference between single genetic defects and multiple genetic defects or with and without combined complement and coagulation mutations were found in this study.

Conflict of interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.03.1623.

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