Complement Mutations in Diacylglycerol Kinase-ε–Associated Atypical Hemolytic Uremic Syndrome

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Abstract

Background and objectives Atypical hemolytic uremic syndrome is characterized by vascular endothelial damage caused by complement dysregulation. Consistently, complement inhibition therapies are highly effective in most patients with atypical hemolytic uremic syndrome. Recently, it was shown that a significant percentage of patients with early-onset atypical hemolytic uremic syndrome carry mutations in diacylglycerol kinase-ε, an intracellular protein with no obvious role in complement. These data support an alternative, complement-independent mechanism leading to thrombotic microangiopathy that has implications for treatment of early-onset atypical hemolytic uremic syndrome. To get additional insights into this new form of atypical hemolytic uremic syndrome, the diacylglycerol kinase-ε gene in a cohort with atypical hemolytic uremic syndrome was analyzed.

Design, setting, participants, & measurements Eighty-three patients with early-onset atypical hemolytic uremic syndrome (<2 years) enrolled in the Spanish atypical hemolytic uremic syndrome registry between 1999 and 2013 were screened for mutations in diacylglycerol kinase-ε. These patients were also fully characterized for mutations in the genes encoding factor H, membrane cofactor protein, factor I, C3, factor B, and thrombomodulin *CFHRs* copy number variations and rearrangements, and antifactor H antibodies.

Results Four patients carried mutations in diacylglycerol kinase-ε, one p.H536Qfs*16 homozygote and three compound heterozygotes (p.W322*/p.P498R, two patients; p.Q248H/p.G484Gfs*10, one patient). Three patients also carried heterozygous mutations in thrombomodulin or C3. Extensive plasma infusions controlled atypical hemolytic uremic syndrome recurrences and prevented renal failure in the two patients with diacylglycerol kinase-ε and thrombomodulin mutations. A positive response to plasma infusions and complement inhibition treatment was also observed in the patient with concurrent diacylglycerol kinase-ε and C3 mutations.

Conclusions Data suggest that complement dysregulation influences the onset and disease severity in carriers of diacylglycerol kinase-ε mutations and that treatments on the basis of plasma infusions and complement inhibition are potentially useful in patients with combined diacylglycerol kinase-ε and complement mutations. A comprehensive understanding of the genetic component predisposing to atypical hemolytic uremic syndrome is, therefore, critical to guide an effective treatment.

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Introduction

Hemolytic uremic syndrome (HUS) is a rare, life-threatening disease characterized by thrombocytopenia, hemolytic anemia, and acute renal failure (1). The most frequent form of HUS follows a diarrheal prodrome and is associated with infections involving shiga toxin–producing *Escherichia coli* strains. Five to ten percent of patients with HUS lack an association with this type of infection. This atypical form of HUS (aHUS) has the poorest long-term prognosis; it is characterized by recurrences and presents a mortality rate approaching 30% (1,2). Genetic analyses in patients with aHUS have revealed that most cases associate with mutations and polymorphisms in complement genes and that the disease develops as a consequence of defective protection of cellular surfaces from complement activation (3–17). The recognition that aHUS is a disorder involving complement-dependent tissue damage provided strong support for the implementation of aHUS therapies based in complement inhibitors (18).

In contrast to this complement-related aHUS, Lemaire *et al.* (19) have reported that as many as 27% of aHUS cases presenting in the first 1 year of life are caused by the deficiency of diacylglycerol kinase-ε (DGK-ε) encoded by the *diacylglycerol kinase-ε* (*DGKE*) gene. It has been proposed that lack of DGK-ε causes enhanced signaling through AA-containing DAGs and results in a prothrombotic phenotype (19,20). Because these patients lacked discernible complement alterations, it was suggested that *DGKE*-associated aHUS represents an alternative mechanism leading to

thrombotic microangiopathy that is independent of complement dysregulation (19).

To get additional insights into this new form of aHUS and estimate the prevalence of carriers of *DGKE* mutations in the aHUS Spanish cohort, we performed analysis of the *DGKE* gene in 83 patients with a disease onset in the first 2 years of life.

Materials and Methods

Patients

Our aHUS cohort includes patients, mainly of Spanish origin, enrolled since 1999. We have performed complement studies that have allowed us to identify mutations, risk haplotypes, and autoantibodies in these patients, most of which have already been published. Currently, our cohort includes 289 patients with aHUS: 262 patients from Spain, 9 patients from other European countries, 4 patients from north Africa (Morocco, Tunisia, and Senegal), 6 patients from the United States, and 8 patients from South America. All these patients were diagnosed at specific centers in the countries mentioned and subsequently submitted to the Spanish aHUS registry for complement functional and genetic analyses. aHUS was diagnosed on the basis of microangiopathic hemolytic anemia and thrombocytopenia defined by an hematocrit <0.3 (30%), hemoglobin level <10 g/dl, serum lactate dehydrogenase level >460 units/L, undetectable haptoglobin level, fragmented erythrocytes in the peripheral blood smear, and platelet count <150,000/μl associated with acute renal failure. The studies reported here have the approval of the Institutional Review Board. Informed consent was provided by all individuals participating in the study according to the Declaration of Helsinki.

Complement Profile Assessment

Serum concentrations of C3 and C4 were evaluated by nephelometry (Siemens, Marburg, Germany). Factor H (FH) and factor I (FI) serum levels were measured by ELISA (21,22). Membrane cofactor protein (MCP; CD46) levels on peripheral blood cells were determined by flow cytometry (8). The presence of anti-FH antibodies and C3 nephritic factor and the functional analysis of FH using the sheep erythrocytes hemolysis assay were performed as previously described (23–25).

Mutation Screening and Genotyping

Exons of the genes encoding factor H (*CFH*), membrane cofactor (*MCP*), factor I (*CFI*), C3 (*C3*), factor B (*CFB*), and thrombomodulin (*THBD*) were amplified from genomic DNA using primers derived from the intronic sequences as described (5,10,11,13,26). Exons of *DGKE* were amplified with the primers described in Supplemental Table 1. Automatic sequencing was performed in an ABI3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems). Copy number variations and genomic rearrangements were assessed by multiplex ligation-dependent probe amplification and custom-designed high-density 8×15,000 oligonucleotide CGH arrays spanning the RCA gene cluster (median resolution=110 bp; AMADID 040193; Agilent Technologies, Santa Clara, CA) (27). *CFH* and *MCP* genotyping was performed as described previously (8).

In Silico Analyses of Mutations

The probability of a genetic variant resulting in structural or functional alterations was calculated using bioinformatics prediction tools that discriminate neutral polymorphisms from amino acid substitutions of likely functional importance. To minimize the possibility of false-positive or false-negative results, we have applied four different computational algorithms publicly available: Sorting Intolerant from Tolerant (28) (SIFT; http://sift.jcvi.org), Polymorphism Phenotyping (29) (PolyPhen; http://genetics.bwh.harvard.edu), Mutation Taster (30) (http://www.mutationtaster.org), and Align-GVGD (31) (http://agvgd.iarc.fr). The SIFT algorithm predicts whether an amino acid substitution affects protein function on the basis of sequence homology among related genes and domains over evolutionary time and the physicochemical properties of the amino acid residues. PolyPhen also incorporates the analyses of sequence conservation and the nature of the amino acid residues involved as well as the location of the substitution within the structure of the protein. By accessing a variety of heterogeneous biologic databases and analytical tools, Mutation Taster is able to identify the missense mutations most likely to have functional effects, such as changes to the transcriptional level and pre-mRNA splicing. Align-GVGD combines the biophysical characteristics of amino acid and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral.

Results

The Spanish aHUS cohort includes 83 patients enrolled between 1999 and 2013 with a disease onset in the first 2 years of life; 62 of these patients do not have a discernible genetic or autoimmune abnormality that helps to explain the development of the disease. We performed the analysis of the *DGKE* gene and found that one of these patients (HUS299) was a homozygote for a frameshift *DGKE* mutation. We also screened 21 patients with early-onset aHUS with identified genetic factors and surprisingly, found 3 additional patients belonging to two unrelated pedigrees carrying compound heterozygous mutations in *DGKE* (Figures 1 and 2, Table 1). We did not find heterozygote carriers of *DGKE* mutations in our aHUS cohort.

Patients with Isolated *DGKE* Mutations

HUS299 is a 4-year-old boy from Morocco who presented with aHUS at 13 months, coincident with a diarrhea episode. He required peritoneal dialysis for 22 days but evolved well without recurrences. Three years after remission, the patient remains asymptomatic, with only a residual microalbuminuria (Table 2, Supplemental Material). HUS299 is homozygous for a frameshift mutation in exon 12 (c.1608_1609del; p.H536Qfs*16) of *DGKE* that results in a truncated DGK-ε protein lacking the last 31 amino acids (Figures 1 and 2, Table 1), which is very likely deleterious. This novel *DGKE* genetic variation was not found in 80 control chromosomes from North African populations, and it is not described in the available databases. Additional genetic analysis in this patient failed to reveal other genetic abnormalities. He was also negative for anti-FH autoantibodies, and his plasma complement protein levels, including C3, were normal (Table 3).

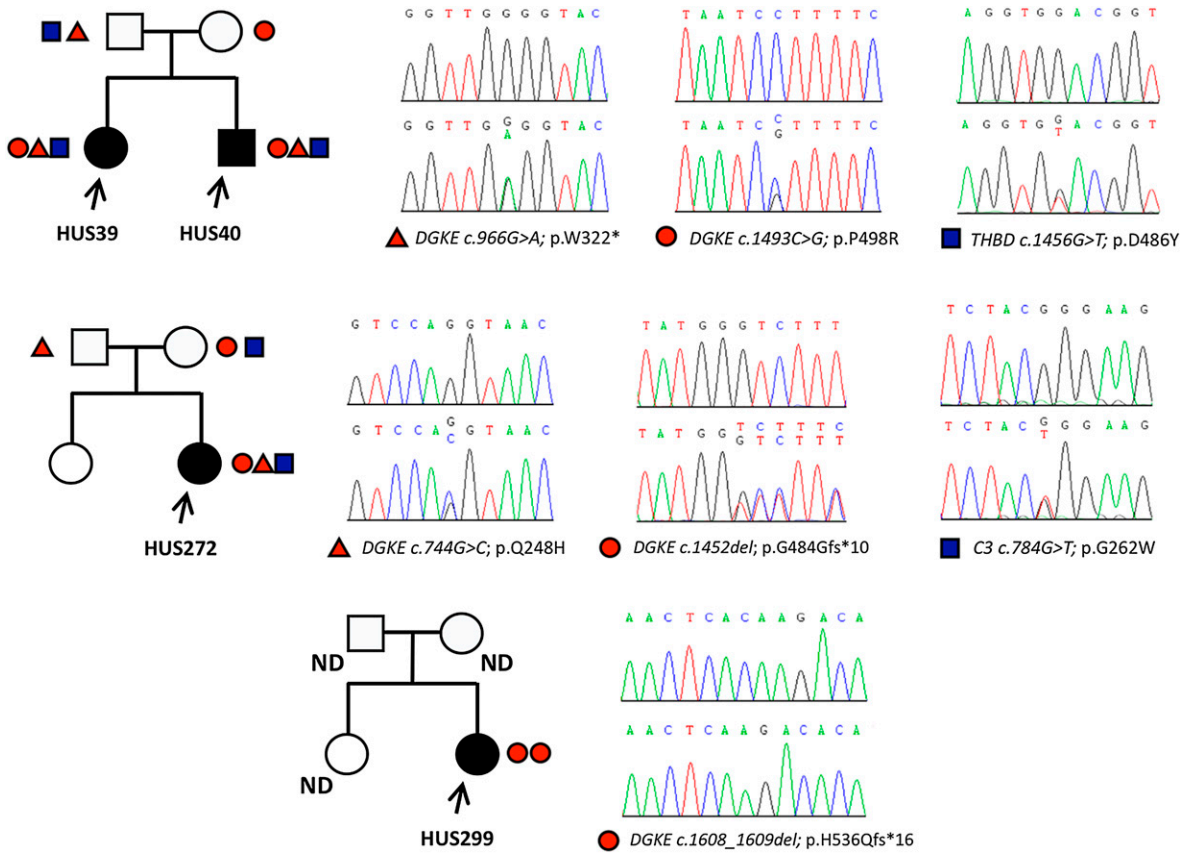

Figure 1. | **Pedigrees of patients with atypical hemolytic uremic syndrome carrying diacylglycerol kinase-ε (*DGKE*) mutations.** For each pedigree, the index patients are indicated with arrows, and the chromatograms for the corresponding mutations are shown. Segregation of the mutations is indicated with colored symbols. HUS, hemolytic uremic syndrome; ND, genetic analyses have not yet been performed.

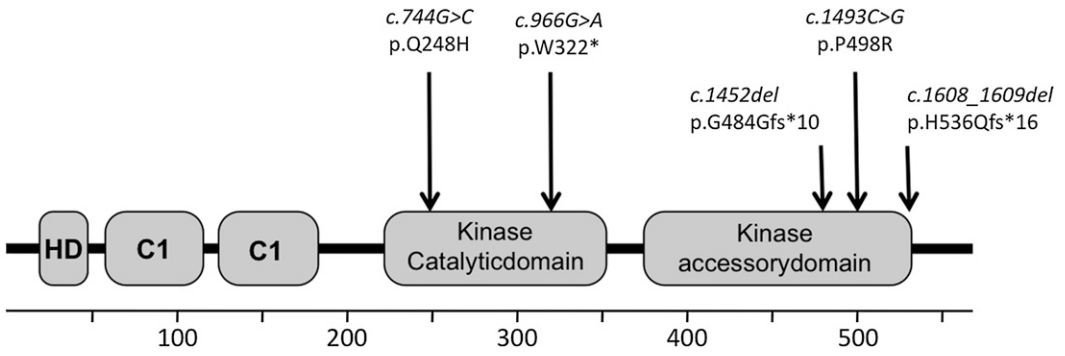

Figure 2. | **Location of the diacylglycerol kinase-ε mutations found in Spanish patients with atypical hemolytic uremic syndrome (aHUS).** A diagram of the diacylglycerol kinase-ε protein depicting the different protein domains is shown. Arrows identify the positions of the five mutations found in the screening of the early-onset Spanish patients with aHUS. C1 domain, kinase catalytic domain and kinase accessory domain; HD, hydrophobic domain.

Patients with Concurrent Mutations in *DGKE* and *THBD*

HUS40 and HUS39 are siblings from an Argentinean family of European ancestry. HUS40 presented with HUS at 7 months of age. Five months later, she had a recurrence that required hemodialysis and continuous ambulatory peritoneal dialysis for a few months. She evolved satisfactorily, partially recovering renal function without dialysis requirement. A third recurrence occurred at 2 years of age that responded well to a protocol of daily plasma infusions, which were suspended 1 year later. She did not suffer any more recurrences. She is now 17 years old and clinically well, with normal blood parameters, serum creatinine of 1.5 mg/dl, and Ccr of 41 ml/min per 1.73 m^2 (Table 2, Supplemental Material). Her brother, HUS39, presented with aHUS at 3 months. He was

Table 1. Diacylglycerol kinase-ε and complement gene mutations found in patients with early-onset atypical hemolytic uremic syndrome

Patient ID	*DGKE*		Other Mutations
	Allele 1	Allele 2	
HUS39	Exon 6; c.966G>A; p.W322*	Exon 11; c.1493C>G; p.P498R	*THBD*; c.1456G>T; p.D486Y
HUS40	Exon 6; c.966G>A; p.W322*	Exon 11; c.1493C>G; p.P498R	*THBD*; c.1456G>T; p.D486Y
HUS272	Exon 4; c.744G>C; p.Q248H	Exon 11; c.1452del; p.G484Gfs*10	*C3*; c.784G>T; p.G262W
HUS299	Exon 12; c.1608_1609del; p.H536Qfs*16	Exon 12; c.1608_1609del; p.H536Qfs*16	None

ID, identification; *DGKE*, diacylglycerol kinase-ε; HUS, hemolytic uremic syndrome.

anuric, requiring acute peritoneal dialysis. On the basis of the experience with his sister (HUS40), he was immediately treated with daily plasma infusions. After 10 days, his renal function improved, and he was discharged, maintaining the daily plasma infusions for 2 months (10 ml/kg per day). Interestingly, when plasma infusions were spaced biweekly, he had an aHUS recurrence that responded well when plasma infusions were returned to daily basis. On the basis of his clinical improvement, plasma infusions were again spaced progressively and finally suspended 5 years later. He is now 11 years old and clinically well, with normal blood parameters, serum creatinine of 0.8 mg/dl, and Ccr of 57 ml/min per 1.73 m^2 (Table 2, Supplemental Material).

HUS40 and HUS39 are compound heterozygotes for a nonsense mutation (c.966G>A; p.W322*) in exon 6 and a missense mutation (c.1493C>G; p.P498R) in exon 11 of the *DGKE* gene (Figures 1 and 2, Table 1). The p.W322* mutation has been described previously as a pathogenic mutation associated with a rare European haplotype (19). Interestingly, the p.W322* mutation in our patients is also associated with the same *DGKE* haplotype (Supplemental Table 2). The second mutation p.P498R is a novel genetic variation that results in an amino acid substitution in exon 11 of the *DGKE* gene, a region that encodes the kinase accessory domain (Figure 2). p.P498R was not found in 140 normal control chromosomes and has not been reported previously in the available *DGKE* mutation databases. We lack assays to test the functionality of the *DGKE* mutations and have no biopsy material from the patients to test DGK-ε expression. However, a very strong argument supporting that p.P498R is a *DGKE* deleterious mutation is that the patients carrying this mutation presented it together with the deleterious p.W322* mutation (patients are compound heterozygotes), which is in agreement with the recessive model of inheritance described for pathogenic *DGKE* mutations. It would be extremely unlikely that this pairing of mutations would have occurred by chance if such infrequent genetic variants were not pathogenic. Furthermore, we have used bioinformatics prediction tools that discriminate neutral polymorphisms from amino acid substitutions of likely functional importance to support that the identified *DGKE* changes represent true disease mutations (Materials and Methods). These *in silico* analyses indicate that p.P498R is a damaging mutation (Table 4).

Genetic analyses in these patients revealed that both patients are also heterozygotes for a mutation in *THBD* (c.1456G>T; p.D486Y) previously found associated with aHUS and shown to be pathogenic (16) (Figure 1). *CFH* and *MCP* genotyping showed that HUS39 and HUS40 are heterozygotes for the *MCP* and *CFH* aHUS risk haplotypes, respectively. The search for anti-FH autoantibodies was negative, and the complement plasma levels were normal, although C3 levels in these individuals, particularly HUS40, were in the lower part of the normal range (Table 3).

Patients with Concurrent Mutations in DGKE and C3

HUS272 is a 4-year-old girl from a German–Spanish family who presented with aHUS at 8 months of age, coincident with an upper respiratory tract infection. She had periorbital edema, low plasma total protein levels, hematuria, and nephrotic proteinuria (Table 2). After 48 hours, she developed generalized edema, hypertension, and thrombocytopenia, and schistocytes were observed at the peripheral blood smear. A biopsy supported the diagnosis of aHUS. She evolved favorably with a progressive decrease of proteinuria levels. Eight months later, coincident with a vaccination, she suffered an episode of oliguria and edema associated with increased proteinuria, which resolved by raising the Enalapril doses. However, 3 months later, after an acute upper tract infection, she had an aHUS recurrence, and her clinical situation deteriorated. C3 was slightly below the normal range. She initiated biweekly plasma infusions, which led to stabilization of the clinical symptoms and near normalization of all blood parameters (Figure 3); however, proteinuria and gross hematuria persisted. One year later, C3 levels dropped to 0.77 g/L, the edema returned, and her clinical situation worsened again. It was decided to switch her from the biweekly plasma infusions to Eculizumab treatment, and thereafter, her clinical situation improved significantly. However, a urine sample revealed ongoing proteinuria. She is currently treated biweekly with Eculizumab, and the clinical situation had improved. Previously, she always had peripheral edema when she had infections; under Eculizumab treatment, however, she remained without obvious edema (Table 2, Supplemental Material).

Table 2. Clinical characteristics of the patients with atypical hemolytic uremic syndrome carrying diacylglycerol kinase-ε mutations

Patient	Sex	Age (yr)	Age at Onset (mo)	Proteinuria Onset Alb (g)/Cr (g)	Thrombocytopenia Onset	LDH (units/L)/ Schisto	sCr onset (mg/dl)	Dialysis Onset	Histology	aHUS Recurrences	Plasma Infusions	Ecu Therapy	Last sCr (mg/dl)	Last Hematuria	Last Proteinuria Alb (g)/ Cr (g)
HUS299	M	4	13	8	Yes	2138/yes	7.7	Yes	ND	No	No	No	0.4	None	0.05
HUS39	M	11	3	>3	Yes	2727/yes	2.8	Yes	ND	Yes (1)	Yes	No	0.8	None	None
HUS40	W	17	7	>3	Yes	4560/yes	4	Yes	TMA	Yes (3)	Yes	No	1.5	None	0.3
HUS272	W	4	8	12	Yes	1032/yes	0.4	No	TMA	Yes (>3)	Yes	Yes	0.3	+++	4.4

Alb, albumin; Cr, creatinine; LDH, lactate dehydrogenase; Schisto, schistocytes; sCr, serum creatinine; aHUS, atypical hemolytic uremic syndrome; Ecu, Eculizumab; M, man; ND, not done; W, woman; TMA, thrombotic microangiopathy.

Genetic analysis showed that HUS272 is a compound heterozygote for a nonsense mutation (c.1452del; p.G484Gfs*10) in exon 11 of *DGKE* that truncates the last 69 amino acids of the protein (most likely deleterious) and a missense mutation (c.744G>C; p.Q248H) in exon 4 of the *DGKE* gene, encoding the kinase catalytic domain of DGK-ε (Figures 1 and 2, Table 1). p.G484Gfs*10 and p.Q248H are novel mutations that were not found in 140 normal control chromosomes and have not been reported previously in the available *DGKE* mutation databases. Similar to the case of the p.P498R mutation found in HUS40 and HUS39, the concurrence of p.G484Gfs*10 and p.Q248H in HUS272 and the results of their analysis *in silico* strongly support that these *DGKE* mutations are pathogenic (Table 4).

Critically, the analyses of all known aHUS genetic risk factors showed that HUS272 carries, in addition to the *DGKE* mutations, a novel mutation in the *C3* gene (c.784G>T; p.G262W) (Figure 1) that, *in silico* analysis, is also clearly identified as a damaging mutation (Table 4). p.G262W was not found in 140 normal control chromosomes and is not included in genetic variation databases. Interestingly, this patient carries the *C3* mutation combined with an *MCP* risk haplotype in homozygosity (Table 3), which is characteristic of patients with aHUS carrying gain-of-function mutations in *C3* and *CFB* (13,32). Complement plasma levels were normal, although C3 levels in this patient were always below or in the lower part of the normal range (Table 3). Notably, her mother, who is also a carrier of the p.G262W *C3* mutation, also presents decreased plasma C3 levels (Figure 3A).

Discussion

Previous studies have identified two groups of patients with mutations in the *DGKE* gene. Ozaltin *et al.* (20) reported nine patients from three consanguineous pedigrees presenting with membranoproliferative GN with histologic signs of both glomerular microangiopathy and endothelial distress at ages ranging from 0.8 to 17 years old. No complement data were reported for these patients (20). A second study by Lemaire *et al.* (19) reported 13 patients from nine unrelated pedigrees also carrying mutations in *DGKE*. In contrast, all these patients presented within the first 1 year of life with aHUS. Notably, these patients belong to a group of pediatric-onset aHUS that was selected to include only patients in whom mutation in known aHUS-associated genes or anti-FH autoantibodies has not been identified (19). Here, we have analyzed the *DGKE* gene in our aHUS cohort and found that 5% (4 of 83) of our early-onset patients carry *DGKE* mutations, including both carriers and noncarriers of mutations in other aHUS-associated genes.

Disease presentation and evolution in our four patients was comparable with that of the patients carrying *DGKE* mutations described by Lemaire *et al.* (19) These patients met the clinical criteria for aHUS at presentation and had disease recurrences within early childhood. Later, one patient clearly progressed to chronic glomerulopathy with hematuria, proteinuria, hypertension, and renal failure, which is distinctive of *DGKE* mutation carriers from other patients with aHUS (Table 2).

Interestingly, we found that three of our patients also carry mutations in other known aHUS-associated genes. Clinical course and severity of the disease in these patients

Table 3. Complement profiles in patients with atypical hemolytic uremic syndrome carrying mutations in diacylglycerol kinase-ε

Patient	Polymorphisms			Complement Assessment[a]					
	CFHR3–CFHR1 Deletion	*CFH* Risk Haplotype	*MCP* Risk Haplotype	FH: 12–56 mg/dl	C3: 90–200 mg/dl	C4: 16–38 mg/dl	FI: 71%–115%	MCP (PBLs)	Anti-FH
HUS299	Het	Het	No	29.7	163	24.2	80	Normal	Neg
HUS39	No	No	Het	31	124	27.4	73	ND	Neg
HUS40	No	Het	No	35.3	87.8	21.6	83	ND	Neg
HUS272	Het	No	Hom	15	86.9	23.6	>100	Normal	Neg

FH, factor H; C3, complement C3; C4, complement C4; FI, factor I; MCP, membrane cofactor protein; PBLs, peripheral blood lymphocytes; anti-FH, autoantibodies against FH; HUS, hemolytic uremic syndrome; Het, heterozygote; Neg, negative; ND, not done; Hom, homozygote.

[a]Complement studies were performed at different times after onset as follows: HUS39, 7 months; HUS40, 6 years; HUS272, 10 weeks; and HUS299, 3 weeks.

were different from those in the patient with isolated *DGKE* mutations. HUS39 and HUS40, who also carry a pathogenic heterozygous mutation in *THBD*, had various aHUS recurrences shortly after the disease onset that responded well to daily plasma infusions. Notably, the course of the disease was more benign in the affected brother (HUS39), who was intensively treated with plasma immediately after the onset of the disease (Table 2). These data point to a potential beneficial effect of plasma infusions in our patients and argue against previous remarks (on the basis of data from patients carrying exclusively loss-of-function *DGKE* mutations) not supporting the use of plasma in affected carriers of *DGKE* mutations (19). Notably, it has been reported that most (88%) patients with aHUS associated with *THBD* mutations responded well to plasma therapy (33). Therefore, we suggest that the association of a *THBD* mutation with the *DGKE* mutations in HUS39 and HUS40 results in a potentially more severe phenotype because of an increased frequency of aHUS recurrences caused by the *THBD* mutation. Consistent with other patients with aHUS carrying *THBD* mutations, our patients responded well to plasma therapy.

HUS272, who in addition to the *DGKE* mutations, also carries a likely pathogenic heterozygous mutation in *C3* and the *MCP* risk haplotype in homozygosity, presented several aHUS recurrences shortly after onset. Biweekly plasma infusions were effective in normalizing blood parameters, and subsequent Eculizumab treatment resolved the infection-associated edemas that were typical in this patient (Figure 3). Compared with the other patients with *DGKE* mutations in this case series, the association of a C3 gene mutation in this particular patient possibly contributed to more severe disease with chronic activation of thrombotic microangiopathy despite plasma treatment and more importantly, a positive response to complement C5 blockage with Eculizumab, which was conducted to remission.

Finally, HUS299, carrying exclusively *DGKE* mutations, recovered renal function without plasma infusions or Eculizumab treatment and has remained stable without recurrences since the aHUS onset 3 years ago (Table 2).

These findings support a potential role of mutations in complement genes in patients carrying *DGKE* loss-of-function mutations. The novelty of our approach is searching for *DGKE* mutations in patients with aHUS with and without mutations in known aHUS-associated complement genes. It is remarkable that, after screening 83 patients with aHUS with a very early onset (<2 years) without excluding those patients with previously identified mutations in known aHUS-associated genes (21 patients), we found 3 patients carrying recessive *DGKE* mutations who also carry mutations in other known aHUS-associated genes and just 1 patient with isolated *DGKE* mutations. These data and the evident correlation of disease recurrences with infection in our patients (Supplemental Material) suggest that the onset and severity of the chronic renal pathology associated with *DGKE* mutations may be influenced by additional complement-related aHUS risk factors. If these data are replicated in other aHUS cohorts and a role for complement dysregulation is finally established in *DGKE*-related aHUS, the suggestion that current treatment on the basis of plasma therapy and complement inhibition are not beneficial in individuals with *DGKE* mutations should, perhaps, be reconsidered, at least for

Table 4. *In silico* analyses of missense mutations

Mutation	PolyPhen (0–1)	SIFT (1–0)	Align-GVGD (C0–C65)	Mutation Taster (0–1)
DGKE; c.744G>C; p.Q248H	0.999 damaging	0 damaging	C15 low risk	0.999 disease causing
DGKE; c.1493C>G; p.P498R	1 damaging	0.05 damaging	C65 high risk	0.999 disease causing
C3; c.784G>T; p.G262W	1 damaging	0 damaging	C65 high risk	0.999 disease causing

PolyPhen, Polymorphism Phenotyping; SIFT, Sorting Intolerant from Tolerant.

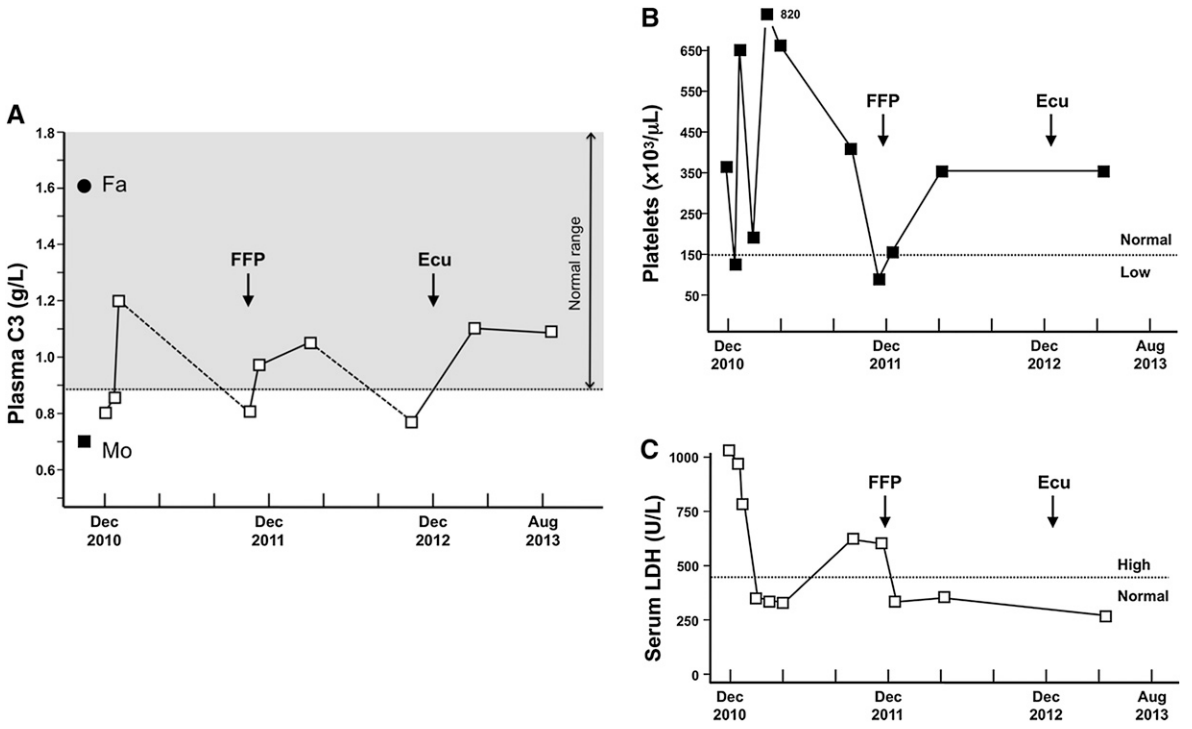

Figure 3. | Evolution of blood parameters in hemolytic uremic syndrome 272 (HUS272). The evolution of the (A) C3, (B) platelets, and (C) lactate dehydrogenase (LDH) serum levels in patient HUS272 from the disease onset are shown. Arrows indicate the start of the plasma therapy (fresh frozen plasma [FFP]) and the Eculizumab (Ecu) treatment. C3 levels in the mother (Mo) and father (Fa) are also indicated.

patients with coexistence of *DGKE* and complement gene mutations. Preventing complement-related thrombotic microangiopathy episodes may result in a more benign course of the renal disease in carriers of *DGKE* mutations.

In conclusion, we found that 5% of the patients in our aHUS cohort who had a very early onset of the disease (<2 years) carry recessive mutations in *DGKE*. Although this percentage is small compared with 27%, which was reported for the French cohort, it is still a significant number. Our patients carry a total of five different mutations in the *DGKE* gene. Four of them are novel mutations, and one (p.W322*) mutation seems to be a relatively prevalent mutation of European origin. The disease presentation and evolution in our patients was comparable with that of patients carrying *DGKE* mutations described by Lemaire *et al.* (19) in the French aHUS cohort. However, in contrast with that previous study, we found that three of our patients carry additional mutations in the known aHUS candidate genes *C3* and *THBD*. Although additional analyses in other aHUS cohorts are needed to replicate our findings, these data suggest that complement-mediated aHUS episodes have a role in modulating the onset and severity of renal disease in carriers of *DGKE* mutations. Finally, this work also illustrates that a comprehensive understanding of the genetic component predisposing to the pathology is critical to guide appropriate diagnostics and effective treatment in aHUS.

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Disclosures

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Supplementary Data

HUS299

HUS299 is a 4y-old male from Marroco who presented with aHUS at 13m-old, coincident with a 7d-long vomiting and diarrhea episode. The analytic parameters at admission were: hemoglobin 7.7g/dL; hematocrit 21.8%; platelets 202,000/μL; LDH 2138U/L; haptoglobin <0.15mg/dL; creatinine 7.7mg/dL and urea 245mg/dL. Schistocytes were present in the peripheral blood smear. He was oliguric and required peritoneal dialysis during 22 days. During that period, anemia progressed and thrombocytopenia dropped to 17,000 platelets/μL, requiring transfusions. Severe hypertension was controlled with Enalapril and Amlodipine. He evolved well and recovered renal function, with only a residual proteinuria of 0.28g/L, discrete anemia (Hg 10g/dL) and normal platelets and serum LDH. Two years after remission, the patient remains asymptomatic and without recurrences. By August 2013 he had normal blood pressure and proteinuria further decreased, remaining only a residual microalbuminuria (MAU/Cr 53.4mg/g). A renal ultrasound at that time was normal.

HUS40 and HUS39

HUS40 and HUS39 are siblings from an Argentinean family of European ancestry. HUS40 is a 17y-old female who at 7mo of age presented an episode of thrombotic microangiopathy. Since STEC-HUS is endemic in that country, it was taken as a typical case evolving satisfactorily. Five months later, however, she had a recurrence that required hemodialysis and continuous ambulatory peritoneal dialysis for few months. aHUS was then suspected and a renal biopsy performed at that time revealed glomerular thrombotic microangiopathy. She evolved satisfactorily from this recurrence, partially recovering renal function without dialysis requirement. A third recurrence occurred at 2y old, following an upper respiratory tract infection. She responded well to a protocol of daily plasma infusions that were spaced progressively and suspended one year later. During the last 14 years she had not suffered any more recurrences. At

the last control, on December 2013, she was clinically well, with normal blood parameters, serum creatinine of 1.5mg/dL and Ccr of 41mL/min/1.73m^2. Her hypertension and residual proteinuria is currently treated with Enalapril and Losartan. HUS39 is a 11y-old male, who in December 2002 presented with aHUS at the early age of 3mo-old.He had severe thrombocytopenia and petechial rush and was anuric, requiring acute peritoneal dialysis. Based on the experience with his sister (HUS40), he was immediately treated with daily plasma infusions. After 10 days, his renal function improved and he was discharged maintaining the daily plasma infusions for two months (10mL/kg/day). Interestingly, when plasma infusions were spaced biweekly he had an aHUS recurrence that responded well when plasma infusions were returned to daily basis. Based on his clinical improvement, plasma infusions were again spaced progressively and finally suspended on December 2007. At the last control, on December 2013, he was clinically well, with normal blood parameters, serum creatinine of 0.8mg/dL and Ccr of 57mL/min/1.73m^2. Like his sister, he is under Enalapril and Losartan.

HUS272

HUS272 is a 4y-old female from a German-Spanish family who in October 2010, at 8mo of age, presented with aHUS coincident with an upper respiratory tract infection. She had periorbital edema, low plasma total protein levels (35g/dL), sCr 0.6mg/dL, Hb 13.4g/dL, 362,000 platelets/μL, hematuria and nephrotic proteinuria (597mg/m^2/h). After 48h she developed generalized edema, hypertension and thrombocytopenia (129,000 platelets/μL) and 6-16% schistocytes were observed at the peripheral blood smear. A biopsy performed on January 2011 revealed the presence of glomerular thrombotic microangiopathy, supporting the diagnosis of aHUS. ADAMST-13 activity was 98.8%. She evolved favorably under Furosemide and Enalapril treatment with a progressive decrease of proteinuria levels. In June 2011, coincident with a vaccination

she suffered an episode of oliguria and edema, associated with increased proteinuria, which resolved raising the Enalapril dosis.

In November 2011, again after an acute upper tract infection, she had an aHUS recurrence and her clinical situation deteriorated. Her blood parameters at that time were: total plasma protein 52g/L, platelets 94,000/μL, Hemoglobin 11.2g/dL, LDH 635U/L, uric acid 7.7mg/dL. Her serum creatinine was 0.4mg/dL, but her cystatin C levels were slightly elevated (1.2mg/L). A kidney ultrasound revealed enlarged, hyperechoic kidneys. C3 was slightly bellow normal range. She initiated biweekly plasma infusions, which after four months led to stabilization of the clinical symptoms and near normalization of all blood parameters (LDH 346U/L, Hb 11.7g/dL, Platelets 350,000/μL, C3 1.06g/L); however proteinuria and gross hematuria persisted.

In November 2012, C3 levels dropped to 0.77g/L, the edema returned and her clinical situation worsened again. It was decided to switch her from the biweekly plasma infusions to Eculizumab treatment and thereafter her clinical situation improved significantly. Her plasma total protein increased to 64g/L, her serum albumin to 42.5 g/L, serum creatinine was 0.18mg/dL, serum cystatin C 0.56mg/dL, LDH 366U/L and C3 0.99g/L. Platelets and LDH were normal (no alteration of these parameters were ever seen again after November 2011, neither under plasma infusions nor Eculizumab treatment). However, a urine sample in May 2013 revealed ongoing proteinuria. She is currently treated biweekly with Eculizumab and the clinical situation had much improved. Previously, she always have had peripheral edema when she had infections, under Eculizumab treatment, however, she remained without obvious edema. Her current values are: serum creatinine 0.3mg/dL, LDH 315U/L, total protein 48g/L, albumin 23g/L, C3 1.32 g/L, 465,000 platelets/μL, Hb 10.9g/dL and Alb(g)/Cr(g) of 4.4.

Supplementary Table 1. PCR primers for *DGKE* exon amplification and sequencing.

Primer Name	Sequence 5' to 3'
DGKE-Exon 2F	CAGCTTGCCCCTGTATGTTG
DGKE-Exon 2R	TCAAAATGAGTTTAGGTCATCAGC
DGKE-Exon 3F	TTCACTGTGCAGATAGTGCAT
DGKE-Exon 3R	TAAATCATTCAGATGCAGGC
DGKE-Exon 4F	AATTTCTGAGGTTCCCTTCC
DGKE-Exon 4R	CTGGGCTGTTTATGCTTTTC
DGKE-Exon 5F	ATAAAGAGTCTGGCAGGGTG
DGKE-Exon 5R	CAGGAGTTTGACAGCAGC
DGKE-Exon 6F	GCACAAGCTTTAGCAAAACA
DGKE-Exon 6R	GAGCATGCAAATTTAGGGAT
DGKE-Exon 7F	GCATGCTCATATACGTGTGG
DGKE-Exon 7R	ACCTAGTGCAGGCTTCTGAG
DGKE-Exon 8cF	TGGACAGTATAAAATAGCCATGTG
DGKE-Exon 8cR	GATCTAATTTGACAACCCTACACA
DGKE-Exon 9F	AAGGAGAATGTGTGCTTCAA
DGKE-Exon 9R	TGTTGAGTCAGGCACTGTATT
DGKE-Exon 10F	TGAGAGGTAGGGAGCATCTT
DGKE-Exon 10R	TTGGGACAAATTAGCCTGTT
DGKE-Exon 11F	AAGTTGATGGTCCAACTGTGTT
DGKE-Exon 11R	CACAATCCCTTAACCCTTATGC
DGKE-Exon 12F	TGCATAAGGGTTAAGGGATTG
DGKE-Exon 12R	TAACTGAAGGCTGGCTGGTT

Supplementary Table 2. DGKE haplotype associated with the W322* mutation.

	rs2235092	**rs7225724**	**rs6503772**		**rs4794670**	**rs11651692**	**rs7209070**
European Haplotype (ref.19)	G	G	A	W322* Mutation	A	G	C
HUS 39	**G**	**G**	**A**		**A**	**G**	**C**
	A	G	A		T	A	C
HUS 40	**G**	**G**	**A**		**A**	**G**	**C**
	A	G	A		T	A	C