Recessive mutations in DGKE cause atypical hemolytic-uremic syndrome

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Pathologic thrombosis is a major cause of mortality. Hemolytic-uremic syndrome (HUS) features episodes of small-vessel thrombosis resulting in microangiopathic hemolytic anemia, thrombocytopenia and renal failure1,2. Atypical HUS (aHUS) can result from genetic or autoimmune factors2 that lead to pathologic complement cascade activation3,4. Using exome sequencing, we identified recessive mutations in DGKE (encoding diacylglycerol kinase ε) that co-segregated with aHUS in nine unrelated kindreds, defining a distinctive Mendelian disease. Affected individuals present with aHUS before age 1 year, have persistent hypertension, hematuria and proteinuria (sometimes in the nephrotic range), and develop chronic kidney disease with age. DGKE is found in endothelium, platelets and podocytes. Arachidonic acid–containing diacylglycerols (DAG) activate protein kinase C (PKC), which promotes thrombosis, and DGKE normally inactivates DAG signaling. We infer that loss of DGKE function results in a prothrombotic state. These findings identify a new mechanism of pathologic thrombosis and kidney failure and have immediate implications for treating individuals with aHUS.

Studies in Mendelian forms of aHUS have implicated mutations in genes of the complement cascade, including those encoding complement factors B (CFB), H (CFH) and I (CFI), complement component 3 (C3), membrane cofactor protein (MCP) and thrombomodulin (THBD)2. All of these mutations result in unrestricted complement activation5. Most are transmitted as autosomal-dominant traits with markedly reduced penetrance; only recessive mutations in CFH and MCP show an apparently high penetrance2. Nonetheless, nearly half of patients with aHUS without secondary causes have no discernable genetic or autoimmune abnormality4.

We studied two unrelated families (kindreds 1 and 2), each with two siblings diagnosed with aHUS in infancy and unaffected unrelated parents. There were no pathogenic mutations in known aHUS genes or any antibodies to CFH in either kindred (Supplementary Table 1). All four affected individuals presented between 4 and 8 months of age with microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure (Table 1 and Supplementary Table 2). Three of the individuals had renal biopsies before age 3, all showing pathology demonstrating chronic thrombotic microangiopathy (Table 1 and Fig. 1). We performed exome sequencing of these four affected subjects (Supplementary Table 3) and identified high-quality variations from the reference sequence, determined their impact on encoded proteins and estimated their allele frequencies.

We posited autosomal-recessive transmission in these families and sought genes with rare homozygous or compound heterozygous variants (minor allele frequency (MAF) <1% and homozygous or...
compound heterozygous genotypes not previously seen in databases) that were shared by both affected subjects in each kindred (Supplementary Table 4). In kindred 1, there was a single, previously unreported homozygous variant that was shared by both affected subjects, and there was one previously unreported, shared compound heterozygous genotype in kindred 2. These previously unreported genotypes occurred in the same gene, DGKE. The first was a homozygous premature termination codon (encoding p.Trp322*), and the second was a compound heterozygote for a frameshift (encoding p.Val163Serfs*3) and a missense mutation (encoding p.Arg63Pro); three unaffected siblings had zero or one of these variants (Fig. 2, Table 1 and Supplementary Fig. 1a).

To extend these findings, we sequenced DGKE in 47 additional unrelated probands with pediatric-onset aHUS and 36 adult-onset aHUS probands in whom there was no mutation in known aHUS-associated genes or CFH antibodies (Supplementary Table 1). From this analysis, we identified six additional index cases harboring rare homozygous or compound heterozygous DGKE variants, all of which had pediatric onset (Fig. 2, Table 1 and Supplementary Fig. 1a).

Parental samples, which were available for all but one kindred, were heterozygous for one of the mutations, with the exception of kindred 5, in which one mutation was apparently de novo. Additionally, kindred 9, independently ascertained in Germany with three affected subjects, showed complete linkage to the DGKE locus (log_{10} odds (LOD) score of 2.53; Supplementary Fig. 1b), and sequencing of all exons in the interval identified a homozygous DGKE mutation (encoding p.Arg273Pro; Fig. 2 and Supplementary Fig. 1a). These nine subjects all met the clinical criteria for aHUS at presentation (Table 1 and Supplementary Table 2). Six had renal biopsies before age 2, all of which read as chronic thrombotic microangiopathy (Table 1 and Fig. 1e–g).

Collectively, the rare DGKE variants found in the nine kindreds included three different premature termination codons, two frameshift mutations, one splice donor-site mutation and two missense mutations that alter conserved residues (Fig. 2 and Supplementary Fig. 1c). Only one of these variants (encoding p.Trp322*) was previously

**Table 1** Demographic, laboratory and clinical characteristics for subjects with DGKE nephropathy

| Subject ID | Age at diagnosis (years) | Gender | Hgb nadir | Evidence of MA | PLT | Cr | Dialysis at onset | Age at diagnosis of first renal biopsy (years) | DGKE alteration 1 | DGKE alteration 2 |
|------------|--------------------------|--------|-----------|---------------|-----|----|-----------------|---------------------------------------------|-------------------|-----------------|
| 1-3        | 0.7                      | M      | 7.2       | Yes           | 50  | 2.84 | Yes             | cTMA (2)                                   | p.Trp322*         | p.Trp322*       |
| 1-4        | 0.3                      | F      | 5.7       | Yes           | 36  | 1.89 | Yes             | ND                                         | p.Trp322*         | p.Trp322*       |
| 2-5        | 0.6                      | F      | 7.3       | Yes           | 35  | 5.32 | No              | cTMA (1)                                   | p.Arg63Pro        | p.Val163Serfs*3 |
| 2-7        | 0.3                      | F      | 6.8       | Yes           | 168 | 1.35 | No              | cTMA (1.5)                                 | p.Arg63Pro        | p.Val163Serfs*3 |
| 3-3        | 0.5                      | F      | 8.4       | Yes           | 132 | 0.62 | No              | cTMA (0.7)                                 | p.Trp322*         | p. Ser11*       |
| 4-1        | 0.3                      | F      | 3.7       | Yes           | 390 | 8.51 | Yes             | cTMA (1)                                   | p.Trp322*         | p.Trp322*       |
| 5-3        | 0.6                      | M      | 7.1       | Yes           | 88  | 5.09 | Yes             | cTMA (1)                                   | p.Trp322*         | p.Trp158Leufs*8 |
| 6-3        | 0.5                      | F      | 9.0       | Yes           | 99  | 2.52 | Yes             | cTMA (1)                                   | p.Gln334*         | p.Gln334*       |
| 7-3        | 0.9                      | M      | 4.9       | Yes           | 125 | 6.79 | Yes             | cTMA (2)                                   | p.IVS5-1          | p.IVS5-1        |
| 8-3        | 0.7                      | M      | 5.0       | Yes           | 214 | 7.19 | Yes             | cTMA (1)                                   | p.Trp322*         | p.Trp322*       |
| 9-3        | 0.3                      | M      | 6.4       | Yes           | 33  | 4.21 | Yes             | cTMA (7)                                   | p.Arg273Pro       | p.Arg273Pro     |
| 9-4        | 0.8                      | M      | 8.2       | ND            | 57  | 0.6  | No              | ND                                         | p.Arg273Pro       | p.Arg273Pro     |
| 9-6        | 0.3                      | F      | 7.3       | Yes           | 32  | 2.21 | Yes             | ND                                         | p.Arg273Pro       | p.Arg273Pro     |

Cr, creatinine; cTMA, chronic thrombotic microangiopathy; Hgb, hemoglobin; MA, microangiopathy; ND, not done; PLT, platelet count.

*Reference values are for infants <1 year of age. **Normal is defined as >0.3 g/dl. ***Microangiopathy diagnosed by high lactate dehydrogenase, low haptoglobin and/or schistocytes on blood smear (details in Supplementary Table 2). Normal is being defined as >10.3 g/dl. Normal is defined as >10.3 g/dl. Subject had many normal platelet counts with acute aHUS. Global renal cortical ischemia and malignant nephroangiosclerosis noted in both kidneys.

**Figure 1** Kidney biopsies of subjects with DGKE mutations show histological features of chronic thrombotic microangiopathy. These include glomerular hypercellularity and split glomerular basement membranes (GBM) by light microscopy and endothelial cell swelling and GBM internal lamina rara widening without electron-dense deposits by electron microscopy. (a,b) Renal biopsy of subject 1-3 at age 2. The image in a shows reduced glomerular capillary lumen, an increased mesangial matrix with mesangial hypercellularity and patchy interstitial fibrosis (Masson’s trichrome). The glomerulus in b shows split GBM (arrow in inset) with debris accumulation in the subendothelial space and a dilated capillary filled with fibrinous material (arrowhead), consistent with a small thrombus (Jones’ stain). (c,d) Renal biopsy of subject 1-3 at age 9 showing the progression of renal damage. The image in c shows a bloodless, markedly lobular glomerulus with extensive fibrosis (Masson’s trichrome), and the image in d shows enhanced global GBM splitting (inset; Jones’ stain). (e–g) Renal biopsy of subject 4-1 at age 1. The image in e shows global thickening of capillary walls, split GBM (arrow in inset), a focal increase in mesangial matrix and a prominent podocyte nucleus (arrowhead; Periodic acid-Schiff). The image in f illustrates split and thickened GBM (Jones’ stain). The electron micrograph in g shows a narrow capillary lumen (L; red line) caused by GBM inner lamina rara expansion (devoid of electron-dense deposits) and hypertrophy of endothelial cells (ECs; black dotted line). Also visible are podocytes (P) with normal (arrow) or effaced (arrowhead) foot processes. Mesangial cell (MC; black line) processes are present between endothelial cells and GBM, consistent with mesangial cell interposition (lead citrate and uranyl acetate). Scale bars, 50 µm (a–f); 10 µm (insets in b,d,e); 1 µm (g).
seen among 8,475 subjects from the National Heart, Lung, and Blood Institute (NHLBI) or Yale exome databases; this variant was heterozygous in two people of European ancestry. The variant encoding p.Trp322* was present in five apparently unrelated subjects with aHUS of European ancestry and was homozygous in three of these five individuals. These three subjects shared an identical and extremely rare haplotype spanning no more than 400 kb at the DGKE locus (Supplementary Fig. 2 and Supplementary Table 3). This indicates a common ancestry for the mutation in each family, with the last common ancestor estimated to have occurred 53 generations ago (95% confidence interval 33–73 generations; Supplementary Fig. 3). The remote shared ancestry of the mutation is consistent with these three families not being closely related.

Twenty-two percent of siblings of index cases in these families (4/18) had aHUS, consistent with recessive transmission with high penetrance. Moreover, rare DGKE variants precisely co-segregated with aHUS in these families, yielding a LOD score of 8.9 (likelihood ratio of 7.9 × 108 in favor of linkage) for complete linkage of these variants to aHUS under a recessive model with rare phenocopies and high penetrance. Similarly, we found no homozygous or compound heterozygous damaging variants (premutation termination, frameshift or splice site variants) among 8,475 subjects in the NHLBI and Yale exome databases (Supplementary Fig. 4). The association of rare recessive genotypes with aHUS was extremely strong (P = 2 × 10−16, Fisher’s exact test). Together these genetic findings conclusively establish recessive loss-of-function mutations in DGKE as the cause of aHUS in these families.

Subjects with DGKE mutations all presented with aHUS in the first year of life (mean 0.5 years, range 0.3–0.9 years; Table 1). DGKE mutations were present in 9 of 22 pediatric patients with aHUS with disease onset before 1 year of age and 0 of 28 patients diagnosed after age 1 year (P = 2 × 10−4). DGKE was a frequent cause of aHUS in the first year of life (13 of 49 cases with aHUS, 27%) and accounted for 50% of familial disease in this age group (three of six kindreds). This uniformly early age of onset defines a distinct subgroup of aHUS (Fig. 3a). The clinical course in subjects with DGKE mutations featured relapsing episodes of aHUS before age 5 years (Fig. 3b).

Abnormal complement activation is a feature of all previously described forms of aHUS. Because DGKE encodes an intracellular enzyme, it is not obvious that complement activation has a role in subjects with DGKE mutations. Detailed assessment of the complement system showed no compelling abnormality in any subject (Supplementary Table 1). Moreover, two subjects with DGKE mutations had aHUS relapses while on anticomplement therapy (eculizumab and fresh frozen plasma infusions, respectively), which are thought to be efficacious in individuals with complement defects (Supplementary Fig. 5 and Supplementary Table 2).

Among subjects with DGKE mutations whose renal function recovered after the onset of aHUS, hypertension, microhematuria and proteinuria persisted in all but one (subject 5–3). In contrast, most individuals with other aHUS subtypes have no residual renal abnormalities between episodes. Progression to chronic kidney disease (CKD) stages 4 and 5 was common by the second decade of life, long after the last acute episode of aHUS (Fig. 3c). Notably, three subjects with DGKE mutations developed nephrotic syndrome 3–5 years after disease onset, a very rare event in other forms of aHUS. Repeat renal biopsies in these subjects were read as chronic thrombotic microangiopathy (Supplementary Table 2). This is probably not a genotypic effect, as two affected siblings have not thus far developed nephrotic syndrome. Treating physicians did not report extrarenal phenotypes.

Three subjects received cadaveric renal transplantation at 2, 19 and 21 years of age using standard pre- and post-transplantation protocols (Supplementary Table 2). Two allografts have survived 2 years and 4 years so far, whereas the other failed after 6 years due to chronic rejection.
Importantly, there have been no aHUS recurrences after transplant. This contrasts with aHUS caused by defects in the soluble complement cascade in which recurrent aHUS is very common and graft failure almost invariably occurs without anticomplement therapy2.

DGKE was first cloned from a human endothelial cell line3; studies have noted high expression in tests and little expression elsewhere4. We examined DGKE expression by protein blotting in human endothelial cells and platelets, the major cell types involved in thrombosis. We found DGKE expression in both (Fig. 4a,b and Supplementary Fig. 6). Moreover, staining of normal human kidney identified DGKE in the endothelium of glomerular capillaries and podocytes (Fig. 4c–g and Supplementary Fig. 7). We found similar results in rat, in which Dgke colocalized with Wt1, a podocyte marker (Supplementary Fig. 8). We established the specificity of this staining in a parallel study of a kidney biopsy from subject 2-7, who is compound heterozygous for a DGKE mutation (Supplementary Fig. 9). We established the specificity of this staining in a parallel study of a kidney biopsy from subject 2-7, who is compound heterozygous for a DGKE mutation (Supplementary Fig. 9).

These findings establish recessive loss-of-function mutations in DGKE as a frequent cause of aHUS in the first year of life. Consanguinity, recurrence among siblings, persistence of hypertension, microhematuria and proteinuria (especially in the nephrotic range), along with the absence of complement abnormalities, suggest the diagnosis of DGKE nephropathy. The molecular diagnosis of DGKE mutation should be straightforward.

DGKE is the first gene implicated in aHUS that is not an integral component of the complement cascade, raising the question of the pathophysiologic mechanism. DGKE preferentially phosphorylates arachidonic acid–containing diacylglycerol (AADAG) to the corresponding phosphatidic acid2. AADAG is a major signaling molecule of the diacylglycerol family and is produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC) in response to cell surface–receptor signaling (Supplementary Fig. 10). AADAGs activate PKC5. In endothelial cells, PKC increases the production of various prothrombotic (von Willebrand factor10, plasminogen activator inhibitor-1 (ref. 11), platelet-activating factor12 and tissue factor13) and antithrombotic factors, such as tissue-type plasminogen activator14 (Supplementary Fig. 10a). What determines the balance between these prothrombotic and antithrombotic factors is poorly understood. AADAG-dependent PKC signaling also drives thrombin-induced platelet activation15 (Supplementary Fig. 10b). Phosphorylation of AADAG to phosphatidic acid by DGKE terminates AADAG signaling. It is therefore plausible that loss of DGKE results in sustained AADAG signaling16, causing a prothrombotic state. This mechanism is supported by experiments with R39022, a small-molecule inhibitor of several diacylglycerol kinases17, including DGKE. This produces platelet activation18 and inhibition of endothelial prostacyclin production19.

A similar mechanism may pertain to podocytes, where DAGs modify slit diaphragm function20, including inducing endocytosis of nephrin21, an effect that could contribute to proteinuria and kidney failure. Additionally, VEGF signaling is essential for podocyte and renal endothelial cell survival, and loss of signaling in the renal endothelium produces thrombotic microangiopathy22,23. PKC-dependent downregulation of VEGFR2 has been reported in podocytes24 and endothelial cells25. This interaction suggests a potential mechanism for the pronounced renal effects of this form of microangiopathy (Supplementary Fig. 10a). Further work will be required to determine the detailed biochemical mechanism(s) linking DGKE deficiency to aHUS.

The specific triggers that account for the episodic nature of acute attacks of aHUS are poorly understood. Dgke-null mice were not reported to have a thrombotic phenotype, perhaps because such an inciting factor was missing26 or because of intrinsic species differences. The universal findings of hypertension, microhematuria and proteinuria, and the unique finding of nephrotic syndrome among
subjects with aHUS who have DGKE mutations, suggests that mutations in DGKE might have a role in other kidney diseases with similar glomerular phenotypes, such as systemic lupus erythematosus–associated glomerulonephritis27, severe pre-eclampsia and HELLP syndrome28, or membranoproliferative glomerulonephritis (MPGN)29. It is currently unclear whether the hypertension, microhematuria and proteinuria in subjects with DGKE mutations requires prior acute episodes of aHUS or whether renal disease can develop without previous aHUS.

In this regard, it is of great interest that an independent study recently reported three families with an early onset MPGN-like syndrome featuring proteinuria, renal failure and reccessive DGKE mutations30. None of the affected individuals in that study was noted to have acute episodes suggesting aHUS; however, histologic features of glomerular microangiopathy were noted. The authors of that study also presented experimental evidence that loss of DGKE in podocytes might contribute to proteinuria and renal damage through aberrant activation of TRPC6.

These findings have immediate implications for the treatment of aHUS. Current guidelines recommend that individuals with aHUS be treated with eculizumab, a C5 antibody that inhibits the complement cascade, or plasma therapy5. The absence of evidence linking DGKE deficiency to the comple ment cascade and relapses of acute aHUS in subjects while receiving these therapies suggests that these treatments may not benefit individuals with DGKE mutations. Moreover, unlike individuals with soluble complement defects, it seems that renal transplantation can be efficacious and safe in individuals with aHUS caused by DGKE mutations, underscoring the importance of diagnosing DGKE nephropathy.

URLs. BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi; ClustalW2, http://www.ebi.ac.uk/Tools/msa/clustalw2/; NCBI protein, http://www.ncbi.nlm.nih.gov/protein; NHLBI Grand Opportunity Exome Sequencing Project (ESP), https://esp.gs.washington.edu/drupal; UCSC Genome browser, http://genome.ucsc.edu/; dbSNP, http://www.ncbi.nlm.nih.gov/snp.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The DGKE variants described in this study are deposited in dbSNP under batch accession number 1058996. mRNA and protein sequences are available at NCBI under the following accession numbers: human DGKE, NM_003647.2; human DGKE, NP_063638.1; cow DGKE, NP_01179859.1; mouse Dgke, NP_062378.1; Xenopus laev is dgke, NP_001087580.1; zebrafish dgke, NP_001165699.1; Drosophila melanogaster dgke, NP_725228.1; and Caenorhabditis elegans dgk-2, NP_001024679.1. The reference protein sequences for pig (NP_001161117; 428 amino acids) and rat (NP_001034430; 407 amino acids) were ~70% shorter than expected.

The full-length pig DGKE protein sequence (564 amino acids) was derived by translating the cDNA AK400222, and the full-length rat DGKE protein (567 amino acids) was reassembled from the mRNA NM_001039341 by removing a short retained intron.

Note: Supplementary information is available in the online version of the paper.

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AUTOR CONTRIBUTIONS

M.L., V.E.-B. and R.P.L. designed experiments and analyzed data. M.C. and R.P.L. developed the exome analysis protocol. S.M.M., J.D.O., J.A. and H.T. directed the exome capture, DNA sequencing infrastructure and information technology. M.C., M.L., R.P.L., G.N. and P.N. performed bioinformatic and statistical analyses. M.L., W.J. and R.P.L. analyzed the age of shared mutation. M.L., W.J. and V.E.-B. performed Sanger sequencing. W.H.T., J.H. and F.F. performed protein blotting experiments. M.L. performed the immunofluorescence studies. M.L.Q. and F.F. performed the immunohistochemistry studies on human kidneys. F.S., S.T., F.N., E.M., D.M., G.D., V.B., B.L., I.C., M.A.M., E.S. and C.L. ascertained and evaluated patients with aHUS. C.L., V.F.-B. and E.S. recruited patients with aHUS. N.R.L., G.W.M. and M.C.G. provided renal pathology expertise. M.L., V.E.-B. and R.P.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

aHUS kindreds. The French aHUS cohort includes 139 affected individuals from 131 unrelated kindreds with pediatric-onset aHUS and 36 unrelated subjects with adult-onset aHUS. These subjects were recruited from 24 participating pediatric nephrology centers from France and one from Belgium; 36 adult nephrology centers also enrolled subjects. This cohort includes 55 affected individuals from 49 kindreds without mutation in any of the known aHUS genes or CFH antibodies. One additional kindred that included three affected subjects without mutations in known aHUS genes was ascertained in Dubai and Germany. Institutional Review Board protocols were approved at all sites involved in the study, and all subjects provided informed consent.

For all subjects, relevant data were abstracted from detailed medical records. Cases with aHUS were defined using the classic diagnostic triad: microangiopathic hemolytic anemia (hemoglobin < 10 g/dl with lactate dehydrogenase > 250 IU/l), haptoglobin < 0.4 g/l or presence of schizontes on blood smear), thrombocytopenia (platelet count lower than the lower limit of normal for age) and renal failure (serum creatinine concentration higher than the upper limit of normal for age). If one of the elements of the diagnostic triad was missing, clear evidence of TMA lesions observed on a kidney biopsy was necessary to substantiate an aHUS diagnosis. Remission was defined by normalization of platelet and lactate dehydrogenase levels, and relapse was defined by recurrence of microangiopathic hemolytic anemia, thrombocytopenia and/or a 25% increase in serum creatinine concentration after at least 2 weeks remission. We specifically excluded individuals with HUS caused by Shiga toxin–producing Escherichia coli and individuals with secondary causes of HUS, such as drug exposure, autoimmune diseases, infections (Streptococcus pneumoniae or HIV), bone marrow or solid organ transplantation, or coagulopathy deficiency.

Assessment of aHUS susceptibility factors. Assessment of complement system function was done at the complement laboratory at Hôpital Européen Georges-Pompidou (Paris, France), which is the national reference center for the evaluation of complement disorders. Blood samples from subjects with aHUS were collected for investigations of complement function and genetic analyses. Similar studies were performed in parallel on samples from 200 unrelated healthy French subjects to generate reference levels and validate any rare variants identified. Extensive investigations of the complement system were performed as described25. Briefly, complement factor H (CFH) and factor I (CFI) antigen were measured by a sensitive ELISA methods using plates coated with mouse monoclonal anti-human CFH (The Binding Site, PC030) and goat polyclonal anti-human CFI (Abcam, ab8843), respectively. The concentrations of factors 3 (C3), 4 (C4) and B (CFB) were measured by nephelometry (Siemens BN ProSpec) using standard procedures and commercially available diagnostic kits (Siemens, OSAP15 (C3), OSAO15 (C4) and OTNS05 (CFB)). MCP surface expression was assessed by flow cytometry of granulocytes exposed to saturating concentrations of phycoerythrin-conjugated mouse anti-human MCP (Serotec, MCA2113PE) or phycoerythrin-conjugated isotypic mouse immunoglobulin G1 (Serotec, MCA928PE). CH50% was assessed with a standard clinical assay. Soluble C5b-9 (sC5b-9) levels were measured with a commercially available ELISA kit using the manufacturer’s protocol (Quidel, A020). The presence or absence of sheep erythrocyte lysis was assessed by adding patient serum to sheep erythrocytes in a solution containing EGTA-Mg buffer (to prevent activation of the classical complement pathway). All subjects were screened for the presence of CFH antibodies using an ELISA plate coated with 0.3 µg of purified human CFH (Calbiochem, 341274). ADAMTS13 deficiency was assessed using standard clinical protocol. All coding sequences of the CFH, CFI, MCP, C3, CFB and THBD genes were sequenced as described25. Screening for unequal crossing over between homologous genes at the THR loci was done with multiplex ligation-dependent probe amplification (MLPA) from MRC Holland.

DNA sequencing and analysis. Genomic DNA from two index cases with aHUS and two affected siblings was prepared and subjected to exome capture using NimbleGen 2.1M human exome capture arrays (Life Technologies) followed by next-generation sequencing on the Illumina sequencing platform as previously described26. Illumina’s processing software ELAND (CASAVA 1.8.2) was used to map reads to the human reference genome (build 19), and SAMtools37 was used to call single-nucleotide variants and insertions/deletions (indels) at targeted bases. Variants with minor allele frequencies <1% in the Yale (1,972 European subjects), NHLBI Grand Opportunity (4,300 European and 2,202 African-American subjects; last accessed November 2012), dbSNP (version 135) or 1000 Genomes (1,094 subjects of various ethnicities; May 2011 data release) databases were selected and annotated for impact on the encoded protein and for conservation of the reference base and amino acid among orthologs across phylogeny. Variants of interest were verified by direct Sanger sequencing. Nomenclature of the DGKE variants is based on NCBI reference sequence NM_003647.

Genomic DNA of two affected members of kindred 9 underwent targeted enrichment of all exons from the interval 49.4–60.3 Mb on chromosome 17 on the basis of evidence of linkage in this family (see below) followed by next-generation sequencing. The targeted capture reagent was prepared by Roche NimbleGen. The analysis prioritized homozygous protein-altering variants in the linked interval that were not present in the Ensembl SNP database, release 54.

To search for DGKE mutations in additional subjects with aHUS, the complete coding region of DGKE was amplified by PCR using specific oligonucleotide primer pairs for each of the 11 exons and subjected to direct Sanger sequencing. Samples from 48 index cases with aHUS but without mutation in the known aHUS genes and 38 index cases with heterozygous variants in known aHUS genes were sequenced.

DGKE variants were identified in exome data from subjects not known to have aHUS from the HapMap CEU and NHLBI exome databases; potential compound heterozygous variants could only be identified in the Yale database because variants in NHLBI are not linked to genotypes.

Origin of the DGKE variant encoding p.Trp322*. SNPs flanking DGKE were selected using Haploview 4.2 (ref. 38). All had MAF > 10% and no violation of Hardy-Weinberg equilibrium (P > 0.05) among European subjects (HapMap CEU). SNPs were genotyped in three apparently unrelated subjects homozygous for the variant encoding p.Trp322* by PCR and direct Sanger sequencing. The boundaries of the homozygous segments were determined for each family by genotyping additional proximal and distal tag SNPs until heterozygous positions were recorded. This defined a shared homozygous haplotype comprising 16 SNPs no more than 400 kb in length.

The population frequency of the shared haplotype was estimated from the frequency of the haplotype in each block of linkage disequilibrium (LD); a frequency of 0.1% was assigned to haplotypes not previously detected in the HapMap CEU data set.

Given the rarity of the haplotype bearing the DGKE variant encoding p.Trp322* in subjects from the CEU population, we examined the ancestry of the siblings from kindred 1 who were homozygous for this mutation by principal component analysis (PCA). All tag SNP genotypes were extracted from exome sequence data using a Perl script that produces PLINK-compatible output files. These SNPs were combined with HapMap data (Phase II release, 2010-08-18). Tag SNPs extracted using PLINK’s LD-based SNP pruning algorithm39 were used as inputs to perform PCA with EIGENSTRAT software (version 3.0)40.

DMLE+2.3 software41 was used to estimate the age of the most common ancestor carrying the DGKE variant encoding p.Trp322*. DMLE+2.3 uses a Bayesian approach to infer the mutation age of a given variant on the basis of evidence from polymorphic markers located within the shared segment. Data for six polymorphic loci spanning the shared segment were entered into the program, and the model whether analyses were performed under a recessive or dominant genetic model. The three unrelated families harboring the mutation whether analyses were performed under a recessive or dominant genetic model. The three unrelated families harboring the mutation were treated as independent chromosomes; however, results were similar when data from six chromosomes were used. Results were also similar whether analyses were performed under a recessive or dominant genetic model. The three unrelated families harboring the DGKE variant encoding p.Trp322* were all of western European ancestry; We estimated western Europe’s population growth rate (PGR) using census data from France with the equation PGR = ln(Tf/T0)/g, where Tf and T0 are the French populations in the years 2009 (62.5 million) and 1806 (21 million), respectively, and g is the number of generations during this period (g = 8.1, assuming ~25 years per generation). With these parameters, the PGR is estimated at 0.09. When using Tf from the 1954 census data (42.8 million; g = 2.2), the PGR is 0.17. To calculate the proportion of sampled chromosomes (PSC), we used the following equation:

\[
\text{PSC} = \frac{g}{g + \text{g}_0}
\]

where \(g\) is the generation length estimated in the previous step and \(\text{g}_0\) is an average generation length of the population, which we have estimated at 25 years.

When we used g = 2.2 years, PSC was 0.09, which is in good agreement with our previous estimate. These results indicate that the mutation arose in the recent European population history and suggests that the mutation spread rapidly in the affected population. This is consistent with the fact that the mutation was detected in a large number of unrelated individuals from different kindreds.
values: the number of chromosomes for patients with DGKE nephropathy harboring the variant encoding p.Trp322* (n = 8), the estimated MAF in CEU subjects (MAF ≤ 0.01% on the basis of empiric data from control exomes) and western Europe’s population (P = 200 million), where PSC = n/(MAF × P × k) chromosomes. A sensitivity analysis was performed with various combinations of PSC and PGR values centered around the estimates described above. The software ESTIAGE was used to ascertain whether the results obtained with DMLE+2.3 were plausible. ESTIAGE uses a likelihood-based method to estimate mutation age from information related to polymorphic markers located within or at the boundaries of the shared homozygous segment. Twenty-four SNPs spanning the DGKE locus were used for input. The mutation rate was set to 2 × 10−8. Examples of input files for both software are available upon request.

Analysis of linkage and association. Genome-wide analysis of the linkage analysis in kindred 9 was performed by comparing the inheritance of SNP genotypes (Affymetrix GeneChip Human Mapping 250K SNP NspI Array) to the inheritance of aHUS in all pedigree members, specifying aHUS as a rare recessive trait with high penetrance and rare phenocopies. Multipoint LOD scores were calculated using ALLEGRO43. Haplotypes were reconstructed with ALLEGRO and presented graphically with HaploPainter44. The significance of rare DGKE variants in all aHUS kindreds was assessed by comparing their segregation to the inheritance of aHUS in the kindreds.

Parameter LOD scores were calculated, specifying aHUS in kindreds with DGKE mutations as an autosomal recessive trait with complete penetrance and zero phenocopies. Fisher's exact test was used to compare the prevalence of recessive DGKE variants among nonconsanguineous index cases of pediatric-onset aHUS to the corresponding prevalence in 8,475 control exomes from NHLBI and Yale.

DGKE protein expression in platelets and endothelial cells. Venous blood was drawn from three healthy adult human volunteers and three wild-type C57BL/6 adult mice at 8 and 18 weeks of age. Platelets were prepared from blood by differential centrifugation as described45. The cytoplasmic and membrane fractions of total protein extracts were harvested by subcellular protein fractionation (Thermo Scientific). Alternatively, whole platelet lysates were prepared by lysis with NP-40 in the presence of protease and phosphatase inhibitors. Whole-cell protein extracts from human embryonic venous endothelial cells were prepared in an analogous fashion.

For protein blotting, 50 μg of cytoplasmic and membrane platelet extracts, and total platelet and endothelial protein extracts were subjected to SDS-PAGE and analyzed by protein blotting. After blocking, the membranes were probed with the following primary antibodies: for mouse extracts, mouse monoclonal anti-DGKE (1:1,000, Sigma, WH008526M3); and for human extracts, rabbit polyclonal anti-DGKE (1:1,000, Abcam, ab96790) or mouse monoclonal anti-DGKE (1:1,000, R&D, MAB5125). Primary antibodies to β-tubulin (1:500, Santa Cruz, H-235) and Na,K-ATPase (1:500, Cell Signaling, 3010) were also used as loading controls and to assess the purity of the cytoplasmic and membrane fractions, respectively. Secondary antibodies were linked to horseradish peroxidase (1:5,000, Thermo Scientific) and incubated with the membrane for 1 h at room temperature.

Microscopy. We stained kidney sections from two adult subjects with perirenal tissue for immunohistochemistry with DGKE and CD34 antibodies. Briefly, paraffin was removed with xylene and alcohol, and heat-induced antigen retrieval was done with 10 mM citrate, pH 6.0. Endogenous peroxidase activity was quenched with hydrogen peroxide. Slides were washed twice with Tris-buffered saline (TBS)-Tween between each of the following steps: after blocking with 5% human serum in TBS, the slides were incubated with primary antibodies for 1 h, biotin-labeled secondary antibody for 1 h and then streptavidin–horseradish peroxidase (Dako, P0397) for 30 min. DAB reagent (Dako) was then applied for 5 or 30 min, and slides were then washed in water. Hematoxylin counterstain was applied to slides before mounting. The primary antibodies included rabbit polyclonal anti-DGKE (1:50, Novus Biologicals, H00008526-M03) and mouse monoclonal anti-CD34 (1:50, Dako, M7165). The immunoperoxidase protocol used for Supplementary Figure 7d.e was similar except for the following modifications: the primary antibody was mouse monoclonal anti-DGKE (1:30, R&D, MAB5125) and was applied overnight at 4°C. Tris–ethylenediaminetetraacetic acid (EDTA), pH 9.0, was used for antigen retrieval; and the PBS-based blocking solution contained 4% bovine serum albumin, 10% normal goat serum and 0.1% Triton X-100.

Kidney specimens were obtained from adult wild-type rats after infusion of PBS and 4% paraformaldehyde and equilibration overnight in 30% sucrose solution before freezing. Cryostat sections were prepared from frozen tissue (thickness, 10 μm) and fixed using the Nakane protocol (Protocol-Lysine-Parafomaldehyde fixation)46. Slides were washed three times with TBS between each of the following steps: after permeabilization with 0.1% Triton X-100, slides were blocked with 10% goat serum, 1% bovine serum albumin and 0.1% Triton X-100 diluted in TBS at room temperature for 1 h. The slides were incubated with primary antibodies at 4°C overnight and then with secondary antibodies for 1 h at room temperature. Slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Vector Laboratories). Primary antibodies used were directed against DGKE (monoclonal mouse, 1,30; R&D, MAB5125) or WTI (polyclonal rabbit, 1:200, Santa Cruz, sc-192). Fluorescent-labeled secondary antibodies (Invitrogen) were used at 1:200.

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