Supplementary Material

Hemolytic uremic syndrome associated gene sequencing

Genomic DNA was extracted from peripheral blood leukocytes following standard procedures. Sequencing of the coding exons and the intronic flanking regions (±10 base pairs) of complement factor H (*CFH*, including variant rs3753394), membrane cofactor protein (*MCP* or *CD46*, including variant rs7144), complement factor I (*CFI*), complement factor B (*CFB*), complement component C3 (*C3*), thrombomodulin (*THBD*) and diacylglycerol kinase epsilon (*DGKE*) was performed by amplicon-based next generation sequencing as described previously [1].

Anti-FH antibody screening

Screening for anti-FH autoantibodies was undertaken using an enzyme-linked immunosorbent assay (ELISA) as described previously [2]. The threshold of positivity was set at mean +2 standard deviation of the values (56 AU/ml) recorded in 98 healthy individuals.

FH serum level measurement

FH serum levels (Supplementary Table 1) were measured by ELISA. Microtiter plates were coated with 0.15µg of purified sheep polyclonal anti-human FH IgG (Abcam). After blocking, serum samples (diluted 1:10,000) or standard FH (purified Human Factor H, Calbiochem) were added in duplicate. After washing, mouse monoclonal anti-human FH IgGs (LifeSpan BioSciences) were added (diluted 1:10,000). After incubation with goat anti-mouse horseradish peroxidase- (HRP-) conjugated IgGs (1:2,000, ThermoFisher), samples were read at 450nm after addition of tetramethylbenzidine (TMB) substrate.

Kidney biopsy examination

Light microscopy. Routine processing pipelines for kidney biopsies were used for specimen preparation. Formalin-fixed, paraffin-embedded kidney biopsy tissue was cut to 3 μm thickness, deparaffinized and stained with hematoxylin and eosin, periodic acid–Schiff reagent and Gomori trichrome.

Immunofluorescence microscopy. Snap-frozen kidney tissue sections (2 μm thickness) were incubated with polyclonal fluorochrome-conjugated antibodies (DakoCytomation, Danemark) to detect immunoglobulins (IgG, IgM, and IgA), complement components (C3 and C1q), fibrin, kappa and lambda light chains.

CFH-CFHR copy number variation analysis

Screening for genomic rearrangements affecting *CFH*, complement factor H-related 1 (*CFHR1*), *CFHR2*, *CFHR3*, and *CFHR5* was performed by multiplex ligation-dependent probe amplification (MLPA) assay using the SALSA MLPA P236-A3 ARMD Kit (MCR Holland) implemented with homemade probes (from n° 10 to 14 of Supplementary Table 2) analyzed in a separate assay and covering the last exons and introns of *CFH* (exon 19, intron 20, intron 21, intron 22, and exon 23) [3]. MLPA probes are listed in Supplementary Table 2. *CFHR4* copy number was evaluated using the multiplex PCR as described previously [4]. Primer pairs for multiplex PCR are listed in Supplementary Table 3.

Analysis of FH and FHR1 protein levels

Five microliters of serum from the patient and his family members carrying the *CFHR1-CFHR4* duplication, from 3 healthy controls homozygous for the wild-type allele (ctr1, ctr2 and ctr3), and from 2 healthy controls carrying the *CFHR3-CFHR1* deletion in heterozigosity (ctr4) or homozigosity (ctr5), were diluted 1:40 in loading buffer (4X Laemmil Sample Buffer, Bio-Rad), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in non

reducing conditions and analyzed by Western Blot. After blocking, membrane was probed with mouse anti-human FHR1 IgG (1:1,000, kindly provided by Prof. Peter Zipfel). The antibody detection was performed after incubation with HRP- conjugated goat anti-mouse IgG (1:5,000, ThermoFisher) and addition of enhanced chemiluminescence (ECL) substrate (Amersham).
In a second step, the same membrane was probed with goat anti-human FH IgG (1:10,000, Calbiochem) followed by incubation with HRP- conjugated rabbit anti-goat IgG (1:10,000, Sigma), to evaluate the amount of FH in each sample as control for protein loading. The amount of each protein band was estimated by densitometry using ImageJ (National Institutes of Health, NIH). For each individual, the ratio between FHR1 and FH was calculated (Supplementary Table 4).

Whole-exome sequencing, homozygosity mapping and filtering of variants

Whole-exome sequencing (WES) was performed in the patient and available healthy relatives (parents and brother). Sequencing and bioinformatics analysis of the raw sequence datasets (up to single nucleotide variant and insertion/deletion calls) were undertaken at BGI Tech Solutions Co., Ltd (Hong Kong) using the SureSelect Human All Exon V5 Kit (Agilent Technologies) and Illumina HiSeq technology. Alignment was performed using BWA software against the reference human genome hg19. Single nucleotide variant (SNV) and insertion/deletion (indel) calling were performed using the GATK software. We annotated SNVs and indels using ANNOVAR software.[5] Since the patient is from a consanguineous family and is likely to have two recessive disease alleles inherited from a common ancestor, homozygosity mapping was also performed using PLINK software,[6] using genotypes from WES data. In addition, phenotype base gene prioritization was performed with Exomiser [7] setting the recessive inheritance mode and the following human phenotype ontology (HPO) terms: hemolytic-uremic syndrome (HP:0005575), hemolytic anemia (HP:0001878), thrombocytopenia (HP:0001873), and acute kidney injury (HP:0001919).

After excluding variants with minor allele frequency (MAF) <1%, there were three segregating homozygous exonic and splicing (±2 base pairs) candidates. Two were synonymous variants and were therefore filtered out. The remaining variant was a transition G→A at position 37,425,991 of chromosome 9 at cDNA position 287 in the glyoxylate and hydroxypyruvate reductase gene (*GRHPR*), leading to an amino acid change Arginine to Histidine at protein position 96 (chr9:g.37425991G>A [NG_008135.1], c.287G>A [NM_012203.1], p.R96H [NP_036335.1]). This variant lay within a 5 Megabases long homozygous block. It was not present in public databases (dbSNP147, 1000 Genomes Project, Exome Variant Server, and Exome Aggregation Consortium) and was predicted to be damaging by several tools (SIFT, Polyphen2, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN). The c.287G>A variant had a CADD score of 35, affected the last base of exon 3 of *GRHPR* and was predicted by GenScan (http://genes.mit.edu/GENSCAN.html) and Human Splicing Finder v3 (http://www.umd.be/HSF/) to alter the splicing donor site. Moreover, it was the top ranked variant by Exomiser (Exomiser score 0.911).

Sanger sequencing (Supplementary Figure 1) was performed to validate WES results using the following primers targeting the *GRHPR* exon 3:

5'- ATAAGCGGTGTCCCCATGATA-3' (forward primer)

5'- CCAGCAGTCAGAAACAGTGG-3' (reverse primer)

C5b-9 deposition on endothelial cells

Human microvascular endothelial cells (HMEC-1), preactivated with ADP 10 μM for 10 minutes, were incubated with serum (diluted 1:2 with test medium, HBSS with 0.5% BSA) from the patient, or his available relatives, or a pool of 20 healthy subjects (NHS), or a positive control (an aHUS patient with a *CFH* mutation), in the absence or in the presence of the complement inhibitor sCR1 (150 mg/ml). The staining on the endothelial cell surface with rabbit anti-human complement C5b-9 was evaluated by confocal inverted laser microscope. The area occupied by the fluorescent staining

was evaluated and expressed as pixels^2 per field analyzed. For each sample, the mean of 15 fields (excluding the lowest and the highest values) was calculated. Results are expressed as means±SEMs. Data were analyzed by ANOVA. P values of <0.05 were considered to be statistically significant.

References

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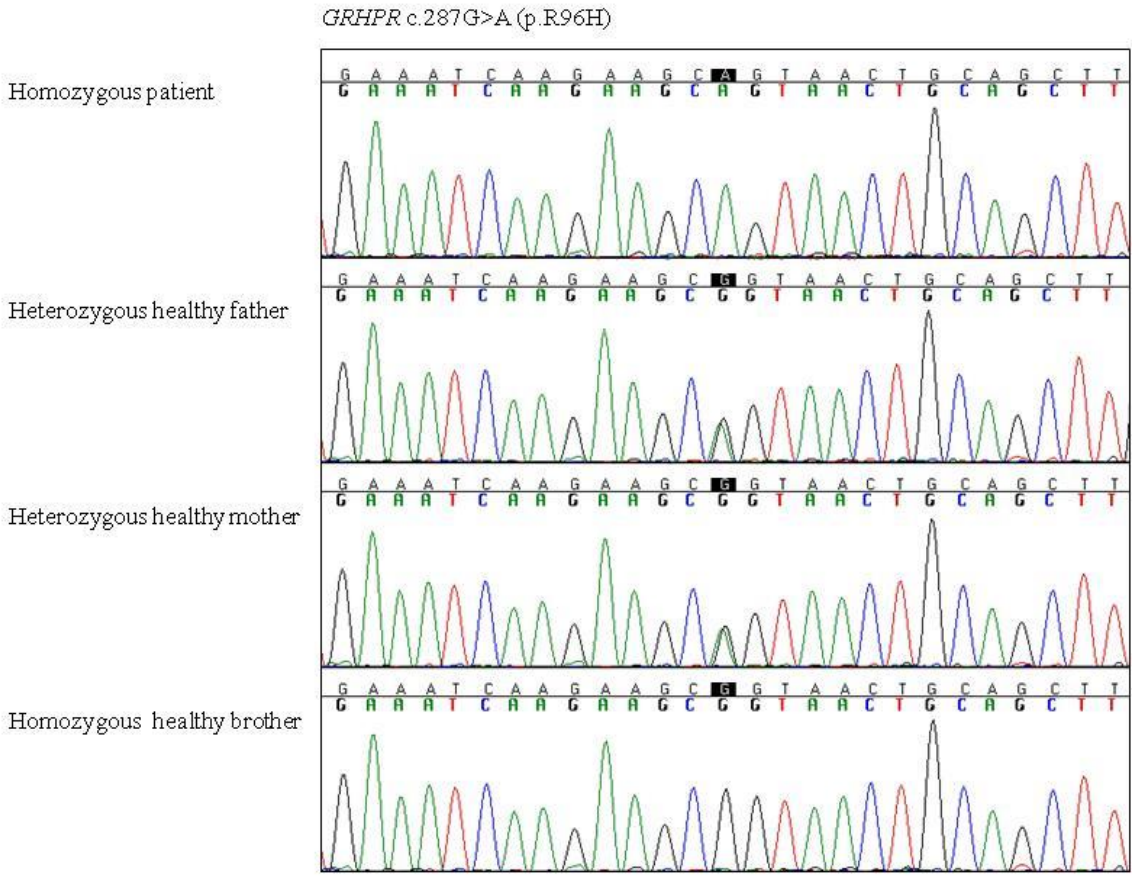

Supplementary Figure 1 illustrates Sanger sequencing electropherograms of the *GRHPR* exon 3 around the c.287G>A variant. The affected child is homozygous for c.287G>A, his healthy parents are heterozygous, and the healthy brother is homozygous for the wild-type nucleotide.

Supplementary Table 1. FH serum levels

Subject	FH concentration (mg/L) (reference range: 173-507)
Patient	316
Brother	269
Father	312
Mother	337

Supplementary Table 2. Probes for MLPA assay

Probe No.	Probe Name	Hybridization Sequence (20 Nucleotides Adjacent to Ligation Site)
1	*CFH* exon 1	TGCTACACAA-ATAGCCCATA
2	*CFH* exon 2	GGTCTGACCA-AACATATCCA
3	*CFH* exon 3	TCCTTTTGGT-ACTTTTACCC
4	*CFH* exon 4	ATTACCGTGA-ATGTGACACA
5	*CFH* exon 6	AAAGAGGAGA-TGCTGTATGC
6	*CFH* intron 10	TAGGTAGTCA-TATTTGGAAC
7	*CFH* intron 12	TGGACACATT-ATGATTGAGT
8	*CFH* exon 13	AGTTGGACCT-AATTCCGTTC
9	*CFH* exon 18	GGAACCATTA-ATTCATCCAG
10	*CFH* exon 19	AGGATGTGTA-TAAGGCGGGG
11	*CFH* intron 20	GAATTCTATT-TACACTTCCG
12	*CFH* intron 21	TAATAGGGTA-TATTATTTTC
13	*CFH* intron 22	GAAAAATCTC-TGTGATGAGT
14	*CFH* exon 23	AGCTTTATTC-GAGAACAGGT
15	*CFH* intron 23	TCAATACATA-AATGCACCAA
16	*CFH* intron 23	CACTTATACA-TGCAATCCGT
17	*CFH* intron 23	AGTCCGAGGT-AGAAAGGGAC
18	*CFH* intron 23	GTGGTAATCT-TGGCTCTCAG
19	*CFHR3* intron 1	AGGTAAGTTA-AAAGAGATCT
20	*CFHR3* intron 1	CATTTTCTTG-TGGAATTACAGC
21	*CFHR3* intron 3	CGGACGACAG-TCTCAGACTT
22	*CFHR3* intron 4	GGGTTATATG-AATTCCTACA
23	*CFHR3* exon 6	TCCCTTCCCG-ACACACTGCTTG
24	*CFHR1* intron 3	AGAGTTTCAG-GTCCATGTGT
25	*CFHR1* intron 5	AATCTGTGAT-TATTTTGTTA
26	*CFHR1* exon 6	CCTGTTCTCA-AATAAAGCTTCT
27	*CFHR1* exon 6	TTTTCCAAGT-TTTAATATGG
30	*CFHR2* intron 1	TGTCTGTACT-TGGAGTTTCG
31	*CFHR2* intron 2	AGATCATAAA-CACTTGATAA
32	*CFHR2* intron 3	AATACCTGTG-TGTGGTTTATAG
33	*CFHR2* exon 4	ATATGCTCCAGG-TTCATCAGTT
34	*CFHR5* exon 1	TGGGTATCCA-CTGTTGGGGG
35	*CFHR5* exon 2	TGAAGAAGAT-TATAACCCTT
36	*CFHR5* exon 3	CTTCAGGACT-AATACATCTG

Supplementary Table 3. Primer pairs for multiplex PCR

Primer Pair No.	Amplicon Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
28	*CFHR4* intron 1	TCTAAACACTCAGCTTCCCTCT	TCTCACAAAATATGCTACTTCTGC
29	*CFHR4* exon 6	CGCGTAGACCATACTTTCCA	ACCCATCTTGTGTGCAGTGA

Supplementary Table 4. *CFHR1* copy numbers and FHR1/FH ratio

Subject	**Number of copies of *CFHR1***	**FHR1/FH ratio**
Patient	4	2.1
Brother	4	1.7
Father	4	2.1
Mother	4	2.9
Ctr1	2	1.2
Ctr2	2	1.4
Ctr3	2	1.0
Ctr4	1	0.5
Ctr5	0	0.0