Complement Factor H Mutation in Familial Thrombotic Thrombocytopenic Purpura with ADAMTS13 Deficiency and Renal Involvement

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Thrombotic thrombocytopenic purpura is a rare disorder of small vessels that is associated with deficiency of the von Willebrand factor–cleaving protease ADAMTS13, which favors platelet adhesion and aggregation in the microcirculation. The disease manifests mainly with central nervous system symptoms, but cases of renal insufficiency have been reported. Presented are findings of the genetic basis of phenotype heterogeneity in thrombotic thrombocytopenic purpura in two sisters within one family. The patients had ADAMTS13 deficiency as a result of two heterozygous mutations (causing V88M and G1239V changes). In addition, a heterozygous mutation (causing an S890I change) in factor H of complement was found in the patient who developed chronic renal failure but not in her sister, who presented with exclusive neurologic symptoms.

J Am Soc Nephrol 16: 1177–1183, 2005. doi: 10.1681/ASN.2005010086

Thrombotic thrombocytopenic purpura (TTP) is a disease of small vessels characterized by anemia that is caused by erythrocyte fragmentation in the microcirculation and thrombocytopenia that is caused by intravascular thrombi of aggregated platelets (1). Recent studies provided substantial evidence that 70 to 80% of cases of TTP are triggered by a deficiency of ADAMTS13 (2–4), a plasma metalloprotease that cleaves von Willebrand factor multimers soon after their secretion by endothelial cells (1,5–7). ADAMTS13 deficiency can be constitutive, as a result of homozygous or double heterozygous mutations in the corresponding gene (8–13), or acquired, as a result of the presence of circulating inhibitory antibodies (1,3,4,14–20).

TTP manifests mainly with central nervous system symptoms, but cases of renal insufficiency have been reported (1). In rare cases, renal involvement is severe enough to cause end-stage renal failure (1,21–25). Those patients' clinical manifestations are difficult to distinguish from those of hemolytic uremic syndrome (HUS), a form of thrombotic microangiopathy characterized by predominant renal involvement, often with renal failure (1,20,26). This difficulty has given rise to a heated debate on whether a severe deficiency of ADAMTS13 activity is enough to distinguish TTP from HUS (27,28).

Here we present findings of the genetic basis of phenotype heterogeneity in patients with congenital ADAMTS13 deficiency. We studied a family with two affected sisters, one who presented with exclusive neurologic symptoms and the other one with severe renal involvement that required chronic dialysis. These diverse clinical manifestations suggested to us that the genetic background could be different.

Materials and Methods

Patients

A woman, now 60 yr old (F48), and her younger sister (F45, died in 2002 at the age of 55 yr) were referred to our International Registry of Recurrent and Familial HUS/TTP in 1996 because of history of recurrent and familial thrombotic microangiopathy. The youngest brother died at the age of 15 yr of leukemia. The other four siblings (three male and one female) all seem to be healthy and have no sign of thrombotic microangiopathy. The two patients had their first episode of the disease at the age of 22 (F45) and 23 yr (F48), respectively, during their first pregnancy and subsequently experienced at least 10 disease relapses concomitant with precipitating events such as pregnancy and spontaneous abortion in the first to second trimester or infection.

In patient F48, neurologic symptoms (dysarthria, dyslalia, aphasia, and facial paralysis) were dominant. Renal function always remained normal, and urine was always negative for proteinuria and hematuria. Remission was achieved with plasma infusions, antiplatelet agents, and anticoagulants, and at present, she is in relatively healthy condition.

In patient F45, the course of the disease was more severe. After a first mild episode of hemolytic anemia and thrombocytopenia during the third month of her first pregnancy (recovered after spontaneous abortion), she had a severe relapse at the age of 23 yr, during the fifth month of her second pregnancy. This was accompanied by confusion, psychomotor agitation, and also coma. She received blood transfusions, steroids, and anticonvulsants. Remission was achieved after spontane-

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1605-1177

ous abortion. After two other spontaneous abortions, each associated with disease relapses, she underwent salpingostomy. Nevertheless, she continued to have relapses, triggered by infection and associated with neurologic signs and acute renal insufficiency. Transient improvement was achieved by plasma infusions, but then renal function progressively deteriorated. At the age of 44 yr, the patient started chronic dialysis. She died at the age of 55 yr because of a cerebrovascular event.

The two patients and all of their available relatives were screened for biochemical and genetic abnormalities of both ADAMTS13 and complement regulatory proteins. One hundred healthy volunteers were also studied as control subjects.

All participants received detailed information on the purposes and design of the study and provided informed written consent, according to the guidelines of the Declaration of Helsinki. The protocol was approved by the institutional review board of the "Mario Negri" Institute for Pharmacological Research.

ADAMTS13 Activity and Antigen

ADAMTS13 activity was measured using the collagen binding assay (20). The protease activity was tested using pooled normal plasma as the source of von Willebrand factor as substrate for ADAMTS13. Human collagen type III (3 μg/ml; Valter Occhiena, Milano, Italy) was used for the collagen binding assay. The values of the protease activity were read from a dose-response curve obtained with reference plasma pool. The lower limit of the assay was 6% of the normal protease levels (20). The presence of ADAMTS13 inhibitory activity was assayed by testing ADAMTS13 activity in mixtures of test plasma and normal pooled plasma at different dilutions (20).

Plasma ADAMTS13 antigen levels were evaluated with an ELISA assay using polyclonal rabbit anti-human ADAMTS13 antibodies. Recombinant human ADAMTS13 was used as standard (29).

Complement Profile

Serum C3 and C4 concentrations were measured by nephelometry. Factor H serum concentrations were assayed by radial immunodiffusion (The Binding Site, Birmingham, UK) (30).

Microsatellite Polymorphism Genotyping

Genomic DNA was extracted either from peripheral blood leukocytes or directly from whole blood according to standard protocols (Nucleon BACC2 kit; Amersham, Little Chalfont, UK). For linkage analysis, we used microsatellite markers flanking complement factor H and membrane co-factor protein (MCP) (31) (chromosome 1q32: D1S240, D1S202, D1S412, D1S2816, D1S413, D1S456, D1S2796, D1S2692) and ADAMTS13 (8) (chromosome 9q34: D9S1847, D9S164, D9S1818, D9S1826, D9S158, D9S1838) genes, identified using the Genome Data Base. Primers were synthesized by Sigma (Sigma-Aldrich, Haverhill, UK). For each PCR reaction, we used 100 ng of DNA in 20-μl final volume that contained 15 pmol of each primer, 16 nmol of dNTP, 2.25 mM $MgCl_2$, 1 U of Taqpolymerase (TaqGold; PE Applied Biosystems, Foster City, CA), and PCR buffer. PCR was performed as follows: 10-min denaturation at 94°C, followed by 35 PCR cycles (94°C for 45 s, 55.5 to 57°C for 45 s, and 72°C for 1 min), and by 10-min extension at 72°C. Amplified DNA samples were mixed with an equal volume of loading buffer, denatured at 75°C for 5 min, and electrophoresed on denaturing 6% acrylamide gel (19:1, acrylamide:bis-acrylamide) in TBE buffer, at 55 W for 2 to 4 h at room temperature. Gels were visualized by silver staining.

Screening for Mutations

Factor H and MCP genes were screened by PCR–single-strand conformation polymorphism (31,32). PCR reactions were done in 20-μl volume that contained 100 ng of DNA, 15 pmol of each primer (Table 1) (31,32), constructed to avoid co-amplification of factor H–related genes, 16 nmol of dNTP, 2.25 mM $MgCl_2$, 1 U of Taqpolymerase, and PCR buffer. Ten-minute denaturation at 94°C was followed by 35 PCR cycles: 45 s at 94°C, 45 s at 55.5 to 57°C, 1 min at 72°C, and a 10-min step at 72°C. Samples were mixed with 20 μl of loading buffer, denatured at 65°C for 10 min, and electrophoresed on nondenaturing 6% (62:1 acrylamide:bis-acrylamide) gel in TAE buffer (pH 6.8) at 35 W for 3 to 5 h at 4°C. Gels were visualized by silver staining. Patients who showed aberrant bands were sequenced.

The complete coding sequence and intronic boundaries (Table 1) of ADAMTS13 gene were analyzed by direct sequencing after purification from agarose gel (1% in TBE) of PCR products (kit QUIAEXII; Qiagen, Hieden, Germany). PCR reactions were performed as above. Amplified DNA was sequenced on both strands using a CEQ8000 XL sequencer (Beckman Coulter, Berkley, CA), following standard protocols. To exclude that mutations were rare polymorphisms, single-strand conformation polymorphism was performed as above on DNA from patients and 100 control subjects.

Results and Discussion

ADAMTS13 activity was <6% (detection limit of the assay) in the two sisters with a diagnosis of TTP. Anti-ADAMTS13 inhibitors were not found, thus excluding an acquired deficiency. It is interesting that complete ADAMTS13 deficiency was also found in a younger brother (F50), even though he had never had any episode of thrombotic microangiopathy. Microsatellite polymorphism genotyping, using polymorphic markers on chromosome 9q34 flanking ADAMTS13 gene, showed a straight correlation between ADAMTS13 activity and haplotype data: The three subjects with complete ADAMTS13 deficiency shared both alleles, whereas subjects with half normal levels shared one allele (Figure 1A). By direct sequencing, a heterozygous G323A missense mutation located in exon 3, which causes a V88M change in the metalloprotease domain, was found in the patients and in their healthy brother with protease deficiency (Figure 2A). In the same subjects, we also found a second heterozygous mutation, a G3777T in exon 27, causing a G1239V change in the first ADAMTS13 CUB domain (8). Neither mutation was found in any of 100 unrelated healthy subjects.

In this family, ADAMTS13 antigen levels paralleled protease activity. The three subjects who carried the two mutations had undetectable ADAMTS13 activity and <10% normal mean plasma antigen levels as measured by ELISA (Figure 1A). These results are consistent with published data showing that most ADAMTS13 missense mutations that are found in patients with TTP result in impaired secretion of the protein (33). Subjects F49 and F51 with a single heterozygous mutation, the G1239V and the V88M, respectively, had approximately half normal protease activity and antigen levels. However, subject F46 without mutation had normal protease activity and antigen levels (Figure 1A).

It has been proposed that a severe deficiency of ADAMTS13 is a beacon for patients with a specific form of thrombotic microangiopathy, labeled as TTP, and could also help in tailoring treatment (27). However, evidence is emerging that different clinical presentations may reflect complex underlying ge-

Table 1. Primers used for ADAMTS13 and factor H mutation screening[a]

	Sense (5′-3′)	Antisense (5′-3′)
ADAMTS13		
exon 1	CCCTGAACTGCAACCATCTT	CAAACCCCAAAGCTGATGTA
exon 2	TCGGTCTCCCCAAGTGTTAG	AACAGGGTTGACAGCAGCTT
exon 3	TCTAGAACCATCGCCCTCTG	CCGAGCCATTCTACCTGAGT
exon 4	ACATGCTGGTGGAGTAGCCTCT	GATGGAGATGCGATGACTTGG
exon 5–6	ACGGGCTAGTCATAGGGTTG	TACAAGGACCCACTGCTTGC
exon 7	GCTGGCGCTGCGGCACTAGGG	GTTGGACGGAGGGGTGGGTTG
exon 8	ACTCCTCCGTCCCGCCTGGTG	GGCCCAGTCAAACAAAAATGT
exon 9	TCTGGGAGGGACAGTTAAGG	TACTGGTCCTGCCTCCTGAC
exon 10–11	GGGTACCCTATGGGTGAGTT	CCTGGTGTGAACCACAGATG
exon 12	GCACTTTTGTCACCCCAGTT	CCAGAGCCTGAACCACTTTG
exon 13–14	CCCAGATGCAAAGGATGAAG	ATCCAGGGCTGAGTGAGTGT
exon 15	TTTTTCCCGACCAGCTAAGA	TCAGAAGTGAGGGCATCTTG
exon 16	CCGGGAAGGAGAGTCACTG	CCCTGTAAGTGACCGCTGA
exon 17–18	GTGATTGCTTGCTGAACGAA	CAGTGTCCTCACCTGCAGAA
exon 19	GAACACCTGGAGAGGCTAGG	ACTTACAACCGCCAGGTGAC
exon 20	GAACCTGCTGGCTGATGAAT	GGATGGTGTTCTTGCTCTGG
exon 21	CACACACGCCACTTCCTG	CCACGTGTTCCCATSTSGTCTG
exon 22	CCATGCGGGCCTTATGTGCTA	TCTGGGTTGCAGTCCTCAAAG
exon 23	TCCCAGCTTCCTGTCTCTTC	TCTCCTGATTCAGCTTTCCAA
exon 24	AGTACACGTGGGTGGAGAGG	CTTTCAGGGGACACGATGAG
exon 25	TTAACTGCCTCCCAGCTTCT	CTTTGCCAGGGAGAAAGAGG
exon 26	CCTCCTGGTCTCCTTCCTCAGCTTGG	TGCCAGGAATGGGGCATGCAGCGTC
exon 27	AGCACCTTGAGCCAACAGGA	CTAGACATACCCGCACTGCAGT
exon 28	CTTGAACTCGGCTCAGTCTACCCTG	TCCCTGGCACGTGCAGACTGA
exon 29	GTTGTCCTGGCCTCTGGCA	GGGTCCCCTAGCCCTGC
Factor H		
promoter	CAAGCACTGCATTCTTGGCA	GCTAGGGAAATTCTCCGTTG
SCR1	CACTTTATGCACTTATTTTGTTTTTATTGTTT	ACACCTAGTTTTCATAAATTTCACAAATG
SCR2A	AGATTCCACTCTACATTGTATGAGAAA	ATGCTAACGTTCTGTTATTTTTTGGT
SCR2B	TCTTAATTATAAACCTCTTTTCGTATGGACTA	TCCTTGCTATTACATACTAATTCATAACTTTTT
SCR3	GGATGATTTTATACATACACATATTTTTCACA	TTCCTTAGAATGAACGATGTTTTAAATG
SCR4	TTTATAAAGATCCAGAAAATAAAGGTAACATTA	ATTATGTCCTGGTCACAGTCCTTTAA
SCR5	TTTAACGGATACTTATTTCTGCATTATCC	TTCAGAATTAAGAAATGGGTCAAGATATG
SCR6	TGACCTAGAAACCCTAATGGAATGT	CACTTATGTGCTCTCCTTTCTTCGA
SCR7	TGAGCAAATTTATGTTTCTCATTTACTTT	TTGCCACAATTAATATAGATGAGTCTTAGA
SCR8	ATAGATATTGAGAATGGGTTTATTTCTGAA	GTTGAGCTGACCATCCATCTTTC
SCR9	TCAGATTGTTTATTAGATGACATTAGAAATGA	CATTCCTTAACAATTCCTCTATATAATTCAAA
SCR10	CAACCTCACTTTATTGTGGCATATG	AAAAGTTGGTATTCAAAGTTCTAATTCTTATTTC
SCR11	TATATTGTAAAACAGACAATTTAACC	ATACAAAATACAAAAGTTTTGACAAG
SCR12	CATCATGTTTTCACAATAAACTTTTTTTG	CTTTGTTAAATGTTGATTTAAGAAGGGTA
SCR13	AACTATTTTTATGTAATAGTTGGTTTGATTCC	GAATACATTTCTGAAAACAAAATAAGAGCTT
SCR14	GTGATAATTTATGAAACAGTTATTG	AGAGAATATTAACCTCATTTGAAAGAATTATGT
SCR15	TTAACCCTTTGATTTTCATTCTTCATT	AACAGAAATTGAGTATTCATATTGGCC
SCR16	GATGTCATAGTAGCTCCTGTATTGTTTATTT	CCACTTACACTTTGAATGAAGAATATTTATC
SCR17	GCCACTGGGCTGGGC	TCTATATATCGCTATTTTAGAATCCATTAGATG
SCR18	CACTTCTTTTTTTTCTATTCAGACACC	AGAATTGAATTTTAAGCACCATCAG
SCR19	TGAAATATCAGACTCATCACAGA	ATACAGTGCTGTGTTTGCG
SCR20A	GTTCTGAATAAAGGTGTGCAC	GCCAAACAGAAGCTTTATTC
SCR20B	CCCCGTTTACACACAAATTCAA	CTACATAGTTGGTTTGGAT

[a]SCR, short consensus repeat.

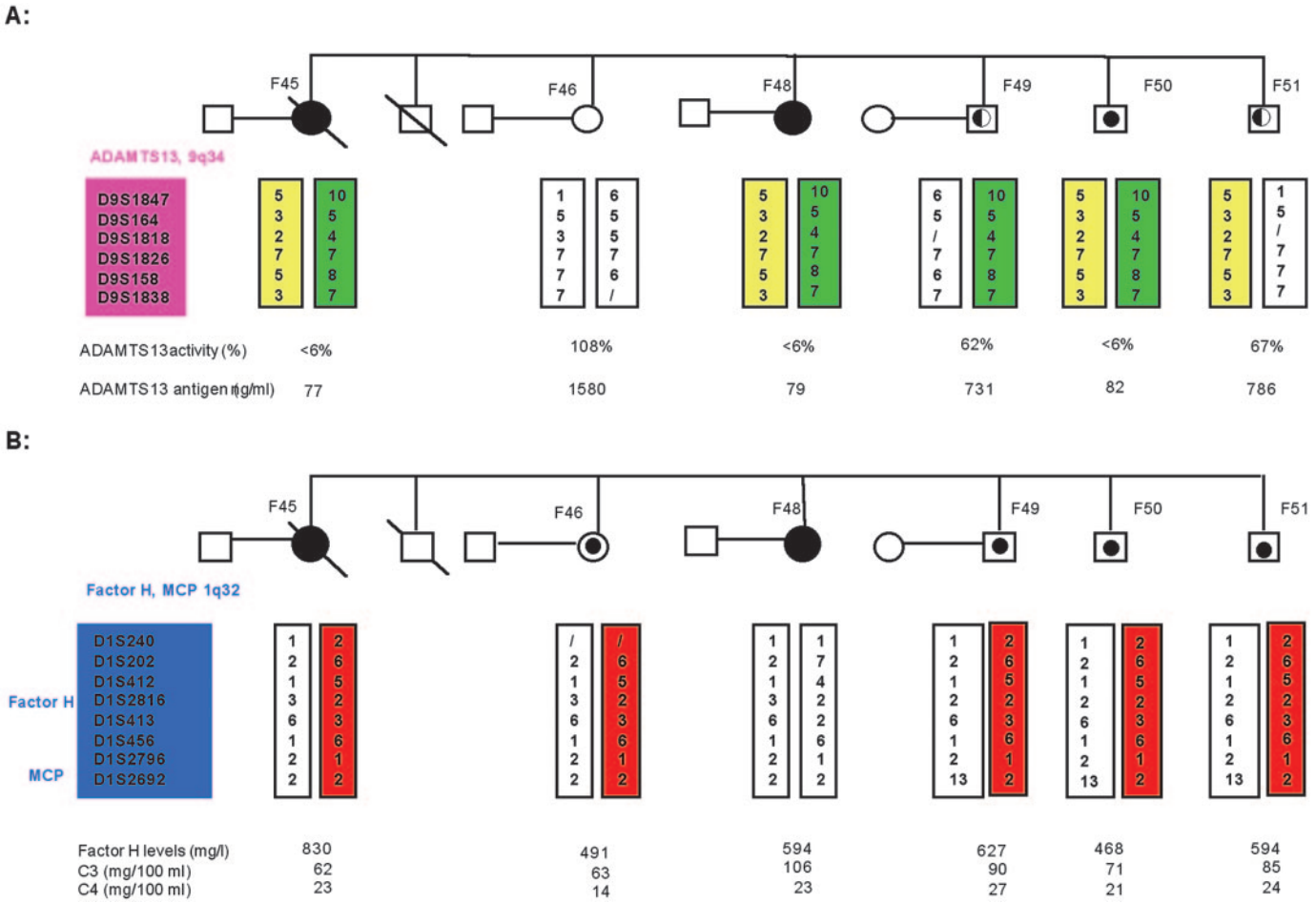

Figure 1. Linkage analysis for chromosome 9q34 (ADAMTS13; A) and chromosome 1q32 (factor H and membrane co-factor protein [MCP]; B) in the pedigree. Circles indicate female individuals; squares indicate male individuals. Affected individuals are indicated by solid symbols, and carriers are indicated by symbols with dots. (A) Six markers (pink square) flanking the ADAMTS13 gene were selected from Genome Data Base for haplotype analysis. Chromosomes that carry the mutations are colored (V88M, yellow; G1239V, green), and normal chromosomes are white. Plasma ADAMTS13 activity and antigen levels are shown below the genotypes. Normal levels, ADAMTS13 activity (range, 50 to 150%); ADAMTS13 antigen (range, 680-1350 ng/ml). (B) Eight markers (blue square) flanking the factor H and MCP genes were selected from the Genome Data Base for haplotype analysis. Chromosomes that carry the mutation are red, and normal chromosomes are white. Serum factor H, C3, and C4 levels are shown below the genotypes. Normal levels: factor H, 350 to 750 mg/L; C3, 83 to 177 mg/100 ml; C4, 15 to 45 mg/100 ml. In the two patients, both ADAMTS13 and complement parameters were measured at remission.

netic abnormalities, because ADAMTS13 deficiency results in a very heterogeneous pattern of clinical manifestations, ranging from no obvious clinical symptoms (9; present article), to prevalent neurologic signs (8–13), to neurologic signs and renal involvement (which may vary from mild urinary abnormalities to severe renal dysfunction requiring dialysis) (20–25). Such heterogeneity can occur even within individuals who carry the same ADAMTS13 genotype as exemplified by the family that we report. In this family, three siblings presented with complete ADAMTS13 deficiency. One had no sign of thrombotic microangiopathy. Another manifested pure neurologic symptoms and responded well to plasma therapy. The third had very severe renal failure and responded poorly to plasma. We hypothesized that modifier genes caused more severe disease and the renal phenotype in the last subject. We focused on genes encoding for the complement regulatory proteins factor H and MCP (34,35), because mutations in those genes have been associated with 30 to 40% familial cases of HUS and result in localized manifestations in the kidneys (31,32,36–39).

The two affected sisters had different genotypes (Figure 1B) on the area of chromosome 1q32, where factor H and MCP genes are mapped. The MCP gene was normal. However, a new heterozygous G2742T mutation in exon 18 (Figure 2B) was found in the factor H gene in patient F45, who developed chronic renal failure, but not in patient F48, who did not. The mutation that was not found in any of 100 unrelated healthy subjects causes a S890I change in short consensus repeat (SCR) 15 of factor H, which was reported recently as a hot spot (in addition to SCR20) for mutations in patients with HUS (40). It is tempting to speculate that in patient F45, factor H haploin-

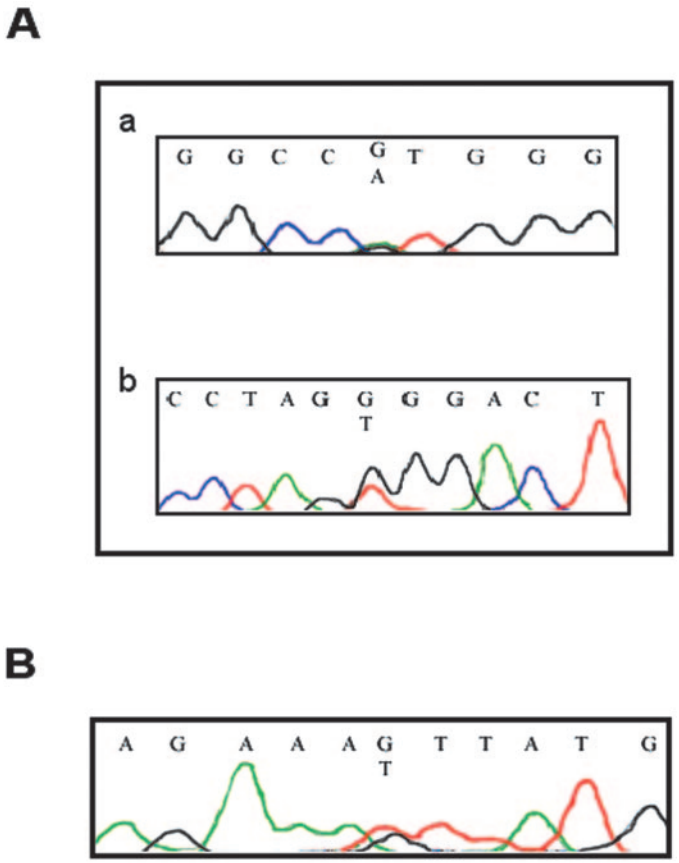

Figure 2. Mutation results. (A) Sequence of ADAMTS13 exons 3 (a) and 27 (b) showing G323A (V88M) and G3777T (G1239V) heterozygous mutations, respectively. (B) Sequence of factor H exon 18 showing a G2742T (S890I in SCR15) heterozygous mutation.

sufficiency caused uncontrolled complement activation and C3b deposition followed by microangiopathic injury in the kidney that superimposed on the systemic thrombotic microangiopathy caused by ADAMTS13 deficiency. Lower than normal serum C3 concentrations and normal C4 levels were found in patient F45, which would indicate that factor H mutation resulted in activation of the alternative pathway of complement (Figure 1B). As expected, patient F48, who does not carry the factor H mutation, had normal C3 and C4 serum concentrations.

It is interesting that the S890I factor H mutation was also found in three unaffected brothers, including F50, who also carries the ADAMTS13 defect, and in an unaffected sister (Figure 1B). In addition, the two patients experienced the first episode of thrombotic microangiopathy in adulthood during pregnancy. Altogether, these data show that ADAMTS13 and factor H gene mutations predispose to thrombotic microangiopathy and organ dysfunction and that triggers including pregnancy and viral or bacterial infections seem to play a relevant role in the full manifestation of the disease.

These results may not explain all cases of renal involvement in patients with TTP and ADAMTS13 deficiency, and additional studies, including a higher number of patients screened for factor H and for other HUS-associated genes, such as MCP and factor I, are required. However, this data disclose for the first time the genetic and phenotypic complexity of TTP and might provide a genetic explanation for cases of clinical syndrome overlapping with HUS.

Acknowledgments

This work was partially supported by grants from Comitato 30 ore per la vita, from Telethon (grants GGP02161 and GGP02162) and from Foundation for Children with Atypical HUS along with The Nando Peretti Foundation. S. Brioschi is a recipient of a fellowship from Fondazione ARMR through a contribution by The Lions Club S. Alessandro Bergamo. S. Bucchioni is a recipient of a fellowship from Fondazione ARMR through a contribution by Anna Vi Gioielleria Bergamo.

We thank Paola Bettinaglio and Francesca Porrati for complement product measurement and Gaia Pianetti for manuscript editing. We are grateful to Edoardo Rossi, Milan, for referring the family to the registry.

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