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BRIEF REPORT

Thrombotic microangiopathy without renal involvement: two novel mutations in complement-regulator genes

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Essentials

- The differential diagnosis among thrombotic microangiopathies (TMAs) is challenging.
- We studied a case of TMA with neurologic symptoms, no renal impairment and normal ADAMTS-13 levels.
- Two novel mutations in complement factor I and thrombomodulin genes were identified.
- Complement-regulator genes can be involved in TMAs with normal ADAMTS-13 regardless of renal damage.

Summary. *Background:* Thrombotic microangiopathies (TMAs) often represent a challenge for clinicians, because clinical, laboratory, and even genetic features are not always sufficient to distinguish among different TMAs. *Objectives:* The aim of this study was to investigate the pathogenetic mechanisms underlying an acute case of TMA with features of both thrombotic thrombocytopenic purpura (TTP) and atypical hemolytic uremic syndrome (aHUS). *Patients/Methods:* We report the case of a 49-year-old woman who developed an acute TMA with neurologic involvement and no renal impairment. ADAMTS-13, von Willebrand factor, and complement-system biochemical characterization was performed on acute phase samples. Exome sequencing and direct Sanger sequencing of previously aHUS-associated genes were performed. The functional consequences of the thrombomodulin (*THBD*) mutation were investigated by *in vitro* expression studies. *Results:* Despite a clinical diagnosis of TTP, the patient had normal ADAMTS-13 levels and increased VWF antigen levels with ultra-large von Willebrand factor multimers. C3, C4, and complement factors H and I (CFI) were normal. Molecular analysis confirmed two novel heterozygous mutations in *CFI* (c.805G>A, p.G269S) and *THBD* (c.1103C>T, p.P368L), and *in vitro* expression studies showed a reduction in the generation of activated thrombin-activatable fibrinolysis inhibitor (TAFIa) caused by mutated *THBD*. This proinflammatory condition, associated with the p.G269S mutation in *CFI*, probably leads to a complement-mediated endothelial activation, with a relevant prothrombotic potential in case of transient environmental triggers. *Conclusions:* This study identified the first case of acute TMA without renal involvement but with neurological damage carrying two novel mutations in complement-regulator genes, highlighting the possible role of the complement system as a common pathogenetic mechanism in TMAs.

Keywords: ADAMTS-13 protein human; atypical hemolytic uremic syndrome; complement factor I; thrombomodulin; thrombotic thrombocytopenic purpura.

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Introduction

Thrombotic microangiopathies (TMAs) are life-threatening diseases characterized by widespread microvascular thrombosis. The clinical distinction between the two main TMAs, thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS), is based on predominant neurological involvement in TTP and more severe

renal injury in HUS [1]; however, this distinction does not seem to be accurate. The pathogenetic roles of the von Willebrand factor (VWF)-cleaving protease ADAMTS-13 in TTP [2] and of complement dysregulation in atypical HUS (aHUS) [3] have helped the differential diagnosis but not in all cases. Severe ADAMTS-13 deficiency is consistent with a diagnosis of TTP but also has been demonstrated in aHUS patients [4]. In addition, almost 20–30% of TTP patients have mild-to-moderate ADAMTS-13 reduction or even normal levels [5–8]. Therefore, currently used clinical criteria and laboratory criteria (i.e., pretreatment ADAMTS-13 activity levels) may not be sufficient to distinguish TTP from aHUS. The diagnostic challenge is made even more problematic by the increasing evidence of complement involvement in TTP [9–12]. Even genetic analysis is not invariably diagnostic: mutations in the genes encoding alternative complement pathway proteins are detectable only in half of aHUS patients [13] and have been recently reported in ADAMTS-13-deficient TTP [14,15].

We hereby report the remarkable case of a patient with clinical features of TTP (neurological signs, no renal involvement) and genetic features of aHUS (mutations in complement-related genes), which might open new horizons in the future approach to TMAs.

Methods

In March 2005, a 49-year-old woman presented to her local emergency department with confusion, fever, epigastric pain, and vomiting, following a flu-like syndrome. Neither relevant medical history nor drug use was reported. Laboratory analyses showed thrombocytopenia (platelets 46×10^9 L^{-1}) and Coombs-negative hemolytic anemia (hemoglobin 8.3 g dL^{-1}, lactate dehydrogenase 1112 U L^{-1}, total bilirubin 1.7 mg dL^{-1}, haptoglobin 0.1 mg dL^{-1}), with schistocytes in peripheral blood smear. There was no apparent sign of renal involvement (glomerular filtration rate 107 mL min 1.73 m^{-2}, normal urinalysis, negative 24-h proteinuria). A diagnosis of idiopathic TTP (based on thrombocytopenia, microangiopathic hemolytic anemia, and neurological injury without renal dysfunction) was made and remission achieved after six plasma-exchanges. Since then, the patient remains relapse free.

Complete ADAMTS-13, VWF, and complement-system biochemical characterization was performed in our center on acute phase samples collected before any plasma treatment.

ADAMTS-13 activity was measured using both collagen binding assay (CBA) and fluorescence resonance energy transfer (FRET) assay, ADAMTS-13 antigen level was measured by ELISA, and anti-ADAMTS-13 antibodies were measured by Western blotting, as previously described [16,17]. VWF:Ag was quantified by using ACL TOP™ Hemostasis Testing System (Instrumentation Laboratory, Badford, MA, USA) with pooled normal plasma. The multimeric pattern of plasmatic VWF was analyzed via sodium dodecyl sulfate agarose gel electrophoresis followed by luminographic visualization of multimers [18].

Complement system activity was evaluated in serum samples by using ELISA (Wieslab complement assay, EuroDiagnostica, Malmö, Sweden). Concentrations of the complement system components C3 and C4 and factor H were measured by radial immunodiffusion using commercial methods. The concentration of complement factor I (CFI) in plasma was measured using an ELISA kit (Uscn Life Science Inc., Houston, TX, USA) according to the manufacturer's instructions. Autoantibodies directed against human factor H were assayed by means of an in-house ELISA method that used purified factor H for capture and antihuman immunoglobulin G (IgG), A (IgA), and M (IgM) for detection [19].

Exome sequencing was performed on an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA), and variants were annotated using dbSNP139 and the Exome Variant Server, while missense mutations were annotated on software that predicts their functional impact, including SIFT and PolyPhen-2. All coding sequences and intron–exon boundaries of previously aHUS-associated genes *CFH, CFI, CD46, CFB, THBD*, and *C3* were also analyzed by direct Sanger sequencing (Applied Biosystem, Foster City, CA, USA).

To evaluate the functional consequences of the thrombomodulin (*THBD*) mutation, pCMV6-entry vectors (Origene, Rockville, MD, USA) containing wild-type or mutated *THBD* cDNA were stably transfected into HEK293 cells. Transfections into HEK293 cells were performed with Lipofectamine 2000 (Thermofisher-Invitrogen, Carlsbad, CA, USA) and selection of transfected stable cells was achieved with 0.5 g L^{-1} neomycin (G418; Invitrogen) after 4 weeks. To confirm the equal expression of recombinant thrombomodulin, both flow cytometry (Abcam, Cambridge, UK) and ELISA (FACSAria, BD Biosciences, San Jose, CA, USA) detection of the membrane protein were performed. The expression of thrombomodulin by FACS was evaluated by incubating the cells with monoclonal mouse antibody against thrombomodulin (R&D systems, Inc., Minneapolis, MN, USA) followed by incubation with the secondary AlexaFluor 488 goat antimouse IgG (Invitrogen). To determine the concentration of thrombomodulin by ELISA (Abcam, Cambridge, UK), membrane proteins were extracted from cell pellets using the Subcellular Protein fractionation kit (Thermo Scientific, Rockford, IL, USA). Total protein concentration in membrane fractions was quantified by using the Bradford assay (BioRad, Hercules, CA, USA). Equal amounts of total membrane proteins were used to determine the THBD concentration. As negative control, HEK293 untransfected cells were used. Cells expressing equal amounts of wild-type or mutant THBD were incubated with 20 μg mL^{-1} of thrombin-activatable

fibrinolysis inhibitor (TAFI) (Haematologic Technologies Inc., Essex Junction, VT, USA) and 4 U mL^{-1} of thrombin. The proteins were analyzed by Western immunoblotting to detect the 35-kDa activated TAFI (TAFIa) fragment by incubating the membranes with a mouse monoclonal antihuman TAFI antibody (Haematologic Technologies Inc.) and then with horseradish peroxidase–conjugated rabbit antimouse IgG (Invitrogen). The luminograms were scanned and the relative amount of the bands was estimated by densitometry using NIH Image J [Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA. http://imagej.nih.gov/ij/. Accessed October 23, 2013].

Results and discussion

ADAMTS-13 activity and antigen levels were normal, with increased VWF antigen levels and the presence of ultra-large VWF (ULVWF) multimers. C3, C4, CFI, and complement factor H levels were normal (Table 1).

Exome sequencing revealed 11 nonsynonymous single nucleotide variants (SNVs) in 12 genes involved in the pathophysiology of TMAs (including SNVs in *DGKE*, recently implicated in aHUS) [20]. Among them, only c.805G>A (p.G269S) in *CFI* and c.1103 C>T (p.P368L) in *THBD* were novel (Table 2). These two variants have been searched in 150 and 474 normal alleles, respectively, confirming them to be rare mutations and not common polymorphisms. This was further confirmed by the absence of the variants in dbSNP139 and Exome Variant Server (with > 3200 European Americans sequenced for *CFI* and > 4300 for *THBD*) databases. Both mutations were predicted to be deleterious for protein function by SIFT, while Polyphen-2 predicted p.G269S to be 'probably damaging' and p.P368L to be 'possibly damaging.' Sanger sequencing confirmed the presence of the heterozygous missense mutations, not previously described in aHUS patients [21–24]. The p.G269S *CFI* mutation causes the change of the small hydrophobic glycine to the hydrophilic serine in the LDLr2 CFI domain. The mutation is close to another mutation reported in aHUS (p.G261D) [25] and, like the latter, was associated with normal CFI levels.

Table 1 ADAMTS-13-, VWF-, and complement system–related plasmatic measurements at acute disease presentation

Parameter	Normal values	Patient's results
ADAMTS-13 activity by CBA, %	46–160	64
ADAMTS-13 activity by FRET, %	50–138	97
ADAMTS-13 antigen, %	40–155	58
Anti-ADAMTS-13 antibodies by Western blotting	Absent	Absent
VWF antigen, %	55–165	344*
ULVWF ratio	0.85–1.21	1.3*
Classic complement pathway activity, %	69–129	72
C3, %	70–130	84
C4, %	70–130	108
CFI, mg L^{-1}	40–80	58
FH antigen, %	66–122	81
Anti-FH antibodies, U mL^{-1}	< 5.20	2.5

CBA, collagen binding assay; FRET, fluorescence resonance energy transfer; VWF, von Willebrand factor; ULVWF, ultra-large forms of von Willebrand factor; C3, complement system component 3; C4, complement system component 4; CFI, complement system factor I; FH, complement system factor H. *Abnormal result.

Of particular interest is the mutation in *THBD*, which encodes thrombomodulin, an endothelial transmembrane glycoprotein that exhibits pleiotropic properties. It is a cofactor for thrombin-mediated activation of TAFI that, when activated to TAFIa, exerts antifibrinolytic and anti-inflammatory activities. TAFIa inhibits fibrinolysis by removing lysine residues from fibrinogen, thereby diminishing the conversion of plasminogen to plasmin. Moreover, TAFIa inactivates several inflammatory mediators, including the complement anaphylatoxins C3a and C5a, via their des-argination [26]. The *THBD* mutation found in our patient is located within the fourth epidermal growth factor (EGF)-like domain, far from mutations previously reported in aHUS [21]. The EGF-like repeats are crucial for thrombin–THBD interaction, with EGF5-6 being required for thrombin-THBD binding, EGF4-6 for protein C activation, and EGF3-6 for TAFI activation.

Because the p.P368L mutation is localized in one of the crucial domains for TAFI activation, we tested whether it determined an altered thrombin-mediated generation of TAFIa. To this end, HEK293 cells stably transfected for equal expression of wild-type and variant thrombomodulin (Fig. 1) were incubated with TAFI and thrombin. Western blot analysis of the cell supernatant showed that the p.P368L thrombomodulin was less effective than wild-type in generating TAFIa (50% reduction vs. wild-type) (Fig. 2). Reduced TAFI activation may result in the impaired degradation and consequent accumulation of complement-derived anaphylatoxins, which are potent inflammatory mediators. By interacting with their receptors C5aR and C3aR, they trigger oxidative burst in macrophages, neutrophils, and eosinophils. C5a is a powerful chemoattractant for macrophages, neutrophils, activated lymphocytes, basophils, and mast cells. Anaphylatoxins may also promote microvascular damage and thrombosis by activating and hurting the endothelium [27]. Of relevance, C5a has been shown to cause rapid expression of P-selectin and secretion of VWF from human cultured endothelial cells (ECs) [28].

Reduced TAFI activation might also lead to enhanced fibrinolysis. However, it has to be considered that TAFIa inhibits fibrinolysis by a threshold mechanism, meaning that fibrinolysis is halted as long as the TAFIa concentration is at or above a threshold value [29]. This implies that a reduction in TAFI activation might not hasten fibrinolysis if the levels of TAFIa remain above the threshold levels.

Table 2 Variants identified by exome sequencing in genes implicated in the pathophysiology of thrombotic microangiopathies (*ADAMTS-13, C3, CFB, CFH, CD46, CFHR1, CFHR3, CFHR4, CFI, DGKE, THBD,* and *VWF*)

Gene name*	Genomic coordinate, chromosome: position	Nucleotide change, reference nucleotide > variant nucleotide	Protein or DNA change†	Present in dbSNP or 1000 Genomes databases‡
CFH	Chr1:196642233	G>A	p.V62I	rs800292
	Chr1:196659237	C>T	p.H402Y	rs1061170
CFHR1	Chr1:196797238	C>T	p.H157Y	rs425757
	Chr1:196797244	C>G	p.L159V	rs113811987
	Chr1:196797292	G>C	p.T175A	rs388862
CFI	Chr4:110678925	T>C	p.T300A	rs11098044
	Chr4:110681504	G>A	p.G269S	Novel§
THBD	Chr20:23029039	C>T	p.P368L	Novel¶
VWF	Chr12:6128443	T>C	p.T1381A	rs216311
	Chr12:6143984	T>C	p.Q852R	rs216321
	Chr12:6153534	T>C	p.T789A	rs1063856

*According to the Human Genome Organization (HUGO) nomenclature. †Predicted changes were checked by Mutalyzer software. ‡rs variant identifying number is reported when the variant is present in the databases. §Not found in a screening of 150 normal alleles. ¶Not found in a screening of 474 normal alleles.

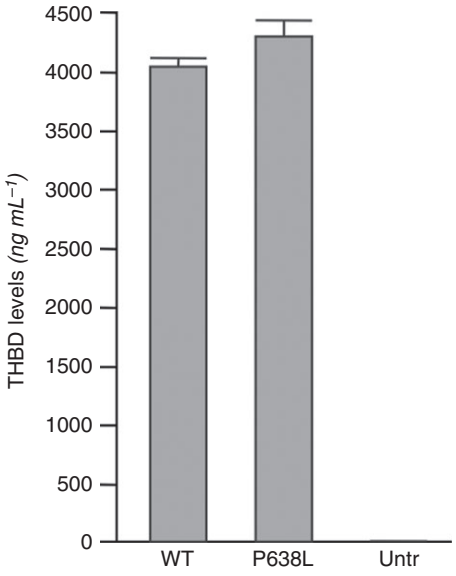

Fig. 1. THBD levels in membrane extracts from HEK293 cells stably expressing wild-type THBD or the P368L THBD variant was determined by ELISA. Equal amounts of membrane total proteins were assessed. Untransfected HEK293 cells were used as negative control. WT, wild type; Untr, untransfected.

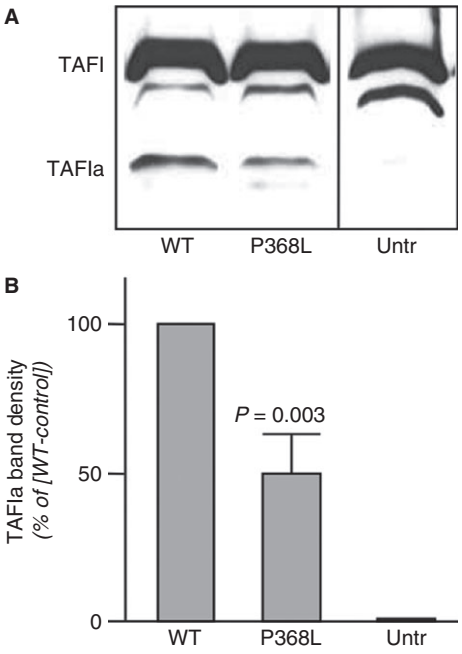

Fig. 2. Thrombin-thrombomodulin–dependent generation of TAFIa (~ 35 kDa) was determined by incubating TAFI (~ 60 kDa) and thrombin on HEK293 cells expressing thrombomodulin (wild-type, mutant [P368L] thrombomodulin, and untransfected HEK293 cells as negative control). Significantly less TAFIa was generated using mutant thrombomodulin. (A) Western blot analysis of the cell supernatant. (B) TAFIa generation determined by densitometric analyses of the band intensities. The results shown are the mean values of four independent experiments. The value of the TAFIa generated with thrombomodulin wild-type in each experiment was taken as 100%. T bar indicates standard error. WT, wild type; Untr, untransfected.

With this background, we assume that a viral infection (flu-like syndrome present in the patient) could have triggered the activation of complement and of vascular ECs in our patient, leading to a prothrombotic state. The excessive C3a and C5a accumulation due to lower TAFIa generation, related to the p.P368L *THBD* mutation, could have also caused a generalized inflammatory response in the microvasculature. Cytokines released during inflammation may alter the kinetics of converting the hyperreactive ULVWF to the smaller and less active plasma forms of VWF through the increased release of ULVWF by activated ECs, which overwhelms ADAMTS-13 cleaving capacity or, alternatively, through direct inhibition of ADAMTS-13 activity [30]. C5a-induced release of ULVWF from microvascular ECs might explain the high levels of VWF antigen and the high ULVWF ratio in our

patient. Of note, at exome analysis there were no genetic variants in either *ADAMTS-13* or *VWF* that could explain this exceptional pattern. The concomitant presence of the p.G269S *CFI* mutation may have resulted in impaired regulation of complement alternative pathway, leading to increased C3 and C5 cleavage to C3a/C3b and C5a/C5b, thus creating a positive feedback loop of inflammation, with additional EC activation. Activated ECs secrete and anchor long strings of ULVWF, prone to initiate platelet adhesion and enhance their aggregation, thus leading to microangiopathy. VWF-related measurements in our patient seem consistent with the proposed mechanism.

In conclusion, our study identified for the first time the unexpected association between mutations in complement-regulator genes and a case of TMA without renal involvement but with neurological damage. Despite the limitation of being a case report, we believe that our study may prompt the evaluation of these mutations in a larger group of patients affected by TMAs and normal ADAMTS-13 activity levels, regardless of the presence of renal injury.

Addendum

F. Peyvandi, R. Rossio, L. A. Lotta, and M. Noris conducted the research. S. Pontiggia, N. Ghiringhelli Borsa, R. Donadelli, and R. Piras performed laboratory analyses. F. Peyvandi, R. Rossio, L. A. Lotta, M. Pizzuti, M. Cugno, R. Donadelli, and M. Noris collected and interpreted the data. F. Peyvandi, R. Rossio, B. Ferrari, L. A. Lotta, and M. Noris wrote the manuscript. All authors approved the final version of the manuscript.

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Disclosure of Conflicts of Interests

F. Peyvandi received honoraria for participating as a speaker at satellite symposia and educational meetings organized by Baxter, Bayer, CSL Behring, Grifols, LFB, and Novo Nordisk; research grant funding was received from Ablynx, Biotest, Kedrion Biopharma, and Novo Nordisk; honoraria for consultancy was received from Ablynx, Alnylam, Biokit, Biotest, Grifols, Kedrion Biopharma, LFB, and Octapharma; and she is member of the Ablynx scientific advisory board. M. Noris received honoraria from Alexion Pharmaceuticals for giving lectures and participating in advisory boards.

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