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Mutation analysis and clinical implications of von Willebrand factor–cleaving protease deficiency

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Mutation analysis and clinical implications of von Willebrand factor–cleaving protease deficiency.

Background. The pentad of thrombocytopenia, hemolytic anemia, mild renal dysfunction, neurologic signs, and fever, classically characterizes the syndrome of thrombotic thrombocytopenic purpura (TTP). TTP usually occurs in adults as an acquired form but a congenital form in children has also been described. In the latter case, the initial presentation is often with neonatal jaundice and thrombocytopenia. The disorder may subsequently take a relapsing course. Deficiency of a recently identified novel metalloprotease, the von Willebrand factor (vWF)–cleaving protease, originating from mutations in the *ADAMTS13* gene plays a major role in the development of TTP.

Methods. Blood for DNA analysis was collected from six unrelated TTP families, consisting of nine patients from four different countries, and was screened for mutations in the *ADAMTS 13* gene. This gene spans 29 exons encompassing ~37 kb. Conventional techniques of DNA extraction, polymerase chain reaction (PCR), and direct cycle sequencing were used.

Results. Eight novel *ADAMTS 13* mutations are presented. Half of the total number of mutant *ADAMTS13* alleles are amino acid substitutions. The disease-causing mutations are spread over the gene. The pathogenicity of the individual mutations is based upon their predicted effect on the ADAMTS13 protein and segregation in family members. Although most of the patients (seven out of nine) had symptoms during the neonatal period, they were in a remarkably good condition. Only one of the nine patients had a decreased glomerular filtration rate (GFR) with proteinuria and hematuria. Another patient had epileptic seizures.

Conclusion. We confirm that deficiency of ADAMTS13 is a molecular mechanism responsible for familial TTP. An early diagnosis allows prophylactic treatment with fresh plasma infusions.

Key words: TTP, ADAMTS 13, vWF-cleaving protease.

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Thrombotic microangiopathies (TMAs) encompass mainly two syndromes: thrombotic thrombocytopenic purpura (TTP) and the hemolytic uremic syndrome (HUS). TTP consists of microangiopathic hemolytic anemia and thrombocytopenia associated with platelet aggregation in the microcirculation responsible for ischemic manifestations [1]. The pathophysiology of TTP involves occlusion of small arterioles and capillaries by platelet plugs containing high quantities of Von Willebrand factor (vWF) [2]. These platelet-rich microthrombi are observed in the small vessels of various organs. vWF is a large glycoprotein essential for platelet adhesion and aggregation, especially at the high shear stress–associated hemodynamic conditions of the microcirculation [3]. vWF is synthesized as a large precursor protein that consists of a 22 amino acid signal peptide, a propolypeptide of 741 amino acids and a mature subunit of 2050 amino acids. Dimers are formed in the endoplasmatic reticulum by covalent dimerization of the subunits at their C-termini. Multimers are formed in the Golgi apparatus by covalent multimerization of the dimers at the D3 domain. vWF is released from endothelial cells as a series of multimers of very high molecular weight. The largest multimers of vWF are the most biologically active. In the plasma, one regulator of the size of the multimers is a specific metalloprotease [4, 5], which cleaves the peptide bond between Y842-M843 of vWF mature subunit [6] and prevents the interaction of the largest multimers with platelets. In plasma of patients with TTP, unusually large multimers of vWF (UlvWF) have been observed [7].

In 1998, a major breakthrough in the understanding of TMA pathology occurred with the discovery of a deficient activity of vWF-cleaving protease (either constitutional or acquired via an autoantibody) specific for TTP [8, 9]. In contrast, vWF-cleaving protease activity was preserved in HUS [10]. Levy et al [11] recently identified mutations in the of *ADAMTS13* gene as the underlying molecular mechanism responsible for familial TTP.

Table 1. Clinical data of the thrombotic thrombocytopenic purpura (TTP) patients included in the study cohort

Patients	1	2[a]	3	4[a]	5	6[a]	7[a]	8[a]	9[a]
Gender	Male	Female	Female	Male	Male	Male	Male	Male	Male
Age of diagnosis *months*	23	21	20	4	7	18	12	9	64
Start of prophylactic plasma infusions	7 years	5 years, 9 months	3 years, 7 months	Treatment symptomatically	7 months, cryoprecipitate	[c]7 years	2 years, 6 months	Treatment symptomatically	[c]9 years
Other affected family members	Possible[b]	+ (brother)	−	+ (brother)	−	+ (brother)	+ (sister)	+ (brother)	+ (brother)
Consanguinity	+	−	−	−	+	−	−	−	−
Symptoms in the neonatal period	+	+	−	+	+	+	+	+	−
Jaundice	−	+	−	+	+	+	+	+	−
Exchange-transfusions	−	+	−	−	−	−	+	+	−
Thrombocytopenia	+	+	−	+	?	+	+	?	−
Rhesus/ABO-incompatibility	−	−	?	?	?	?	−	?	−

[a] Patient 2 and patient 7 are siblings, as well as patient 4 and patient 8 and patient 6 and patient 9, respectively
[b] Patient 1. Brother died immediately after birth. The autopsy revealed widespread thrombi, especially in the myocard, adrenal gland, kidney, and lungs
[c] Prophylactic treatment with hemate (= vWF/factor VIII concentrate) in these patients is described previously [25, 26]. These patients were treated with fresh-frozen plasma before the treatment with hemate started.

ADAMTS13 is a newly identified member of the ADAMTS family of protein-cleaving proteinases. Cleavage of endothelial cell derived ULvWF multimers by ADAMTS13 is a rapid physiologic process that occurs on the endothelial cell surface.

In this article, we report the identification of eight ADAMTS13 mutations in a cohort of nine patients, including three affected sibling pairs from different geographical origins. The presumptive effects of these mutations on the function of the ADAMTS13 protein are discussed. We did not observe a hotspot for mutations in the *ADAMTS13* gene. Finally, our findings suggest genetic heterogeneity in familial TTP.

METHODS

TTP families

Our study cohort consisted of nine children. All patients were diagnosed as TTP. The clinical diagnosis of TTP was based on the presence of hemolytic anemia with fragmented erythrocytes, and thrombocytopenia, which could be accompanied by fever and decrease of GFR. All patients demonstrated a lack of vWF-cleaving protease. The assay of vWF-cleaving protease activity was performed as described previously [12]. Briefly, diluted citrated plasma was activated by barium chloride. This activated plasma was added to protease- free vWF. The reaction was stopped by addition of ethylenediaminetetraacetic acid (EDTA). The extent of vWF degradation was assayed by multimer analysis using sodium dodecyl sulfate (SDS)-electrophoresis in 1.4% agarose gels. Following electrophoresis, the proteins were electrotransferred to nitrocellulose, and vWF was visualized with peroxidase-conjugated rabbit antibodies against human vWF. Patients 1, 2, and 7 were tested for vWF-cleaving protease in our hospital. Patients 3, 6, and 9 were tested by M. Furlan [4] with the same technique. Patients 4, 5, and 8 were tested for the deficiency at the hospital of origin.

Mutation analysis

Genomic DNA was extracted by a salting out procedure from peripheral blood lymphocytes [13]. Sequence analysis was by polymerase chain reaction (PCR) and direct cycle sequencing. Cycle sequencing was carried out by the Dye-deoxy Terminator method, using the ABI Prism 377 sequencer (PE Applied Biosystems, Nieuwekerkaan de Ijssel, The Netherlands) following standard procedures [14]. Primers were from Life Technologies (Breda, The Netherlands). They were designed to permit analyses of complete exons, including intron boundaries, according to the public available sequence (AY 055376). The primer sequences and the PCR protocols are available upon request. To confirm the mutations, restriction enzyme analysis with *BsaJI* (for exon 7 D235H), *AlwNI* (for exon 7 6 bp deletion), *RsaI* (for exon 8), *MboII* (for exon 9) and *AluI* (for exon 12) was performed according to the manufacturer's recommendation. The segregation of mutations has been studied in members of the family of patients 1, 2, 3, 4, and 6.

RESULTS

Clinical and biochemical data

Clinical data were available for nine patients, consisting of three sibling pairs and three cases with no familial history of TTP. The diagnosis of TTP was made between the age of 4 and 64 months. Seven patients (including two affected sibling pairs) had symptoms of jaundice or thrombocytopenia during the neonatal period, while three required neonatal exchange transfusion (Table 1). These early symptoms are likely to represent the first symptoms of TTP. In Table 2 the current situation of the children is described. Surprisingly only one of the patients had a decreased glomerular filtration rate (GFR).

Mutation detection in the *ADAMTS13* gene

Human genomic DNAs from six TTP families, including nine patients (three affected sibling pairs), have been

Table 2. Current situation of the thrombotic thrombocytopenic purpura (TTP) patients

Patients	1	2[a]	3	4[a]	5	6[a]	7[a]	8[a]	9[a]
Current situation									
Age *years*	11.5	15.5	18	4.5	17	13	11.5	7	15
Renal function									
Glomerular filtration rate *mL/min/1.73 m²*	60	148	108	138	107	97	131	133	135
Proteinuria	0.4 g/L	—	—	—	—	—	—	—	—
Hematuria	Microscopic	—	—	—	—	—	—	—	—
Hypertension	—	—	—	—	—	—	—	—	—
Neurological symptoms	—	—	Epileptic seizures	—	—	—	—	—	—
Other (ischemic) symptoms			—		—	—	Right Legg-Calves-Perthes	—	—

[a]Patient 2 and patient 7 are siblings, as well as patient 4 and 8 and patient 6 and 9, respectively

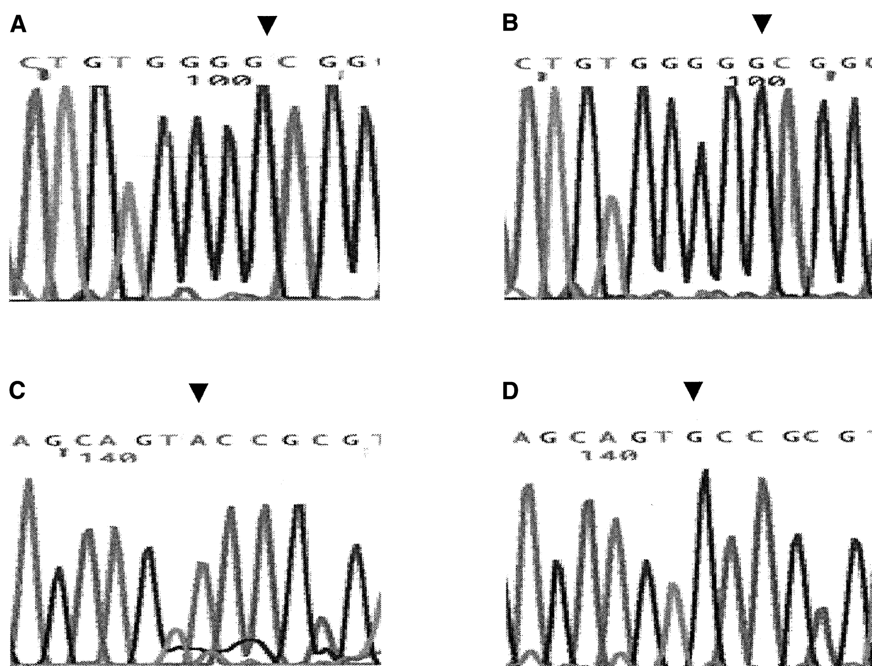

Fig. 1. ***ADAMTS 13*** **mutations in two patients.** A deletion in exon 19 (*A*) of patient 5 and the wild-type of exon 19 (*B*) are shown on top. The mutation found in exon 8 of patient 1 (*C*) and the wild-type exon 8 (*D*) are shown on the bottom.

screened for mutations in the *ADAMTS13* gene by PCR in combination with DNA sequencing of 29 exons that encode the complete coding region. A total of eight different mutations have been identified consisting of four missense mutations, two deletions, one insertion, and one nonsense mutation (Fig. 1 and Table 3). All mutations are novel molecular variants of the *ADAMTS13* gene. In addition, the mutations are spread over the gene (Fig. 2).

In our total study group of six unrelated families, four carry homozygous *ADAMTS13* mutations (patients 1, 2, 5, and 6) and two are compound heterozygotes (patients 3 and 4). During the mutational analyses in the *ADAMTS13* gene, 10 polymorphisms were observed. These appeared to be present in control alleles with variable frequency and have previously been described at NCBI (*http://www.ncbi.nlm.nih.gov*).

The six base pair deletion observed in one allele of patient 4 (and patient 8) caused the removal of two highly conserved amino acids of the ADAMTS 13 protein. The mutation in the other allele of patient 4 (and patient 8) predicts a truncated ADAMTS 13 protein. The homozygous deletion in patient 5 and the homozygous insertion in patient 6 (and patient 9) result in a frameshift and a premature stop codon. The remaining four mutations all result in nonconservative amino acid substitutions and all occur at positions that are perfectly conserved between the human and mouse genes. The parents of the patients in families 1, 2, 3, 4, and 6 were all heterozygous carriers of one mutant *ADAMTS13* allele. All TTP patients displayed homozygous or compound heterozygous mutations in the *ADAMTS13* gene. In addition, in all patients with homozygous or compound heterozygous mutations clinical symptoms were evident. Heterozygous carriers were without symptoms. Fifty control chromosomes (one hundred alleles) were tested for the presence of the four missense mutations that have been identified in our study group. All appeared to be absent in these control chromosomes.

Table 3. *ADAMTS13* mutations identified in thrombotic thrombocytopenic purpura (TTP) patients

Patient	Exon	Mutation at nucleotide	Homo-heterozygous	Predicted effect on protein
1	8	932G→A	Homozygous	C311Y
2 + 7	7	703G→C	Homozygous	D235H
3	9	1058C→T	Heterozygous	P353L
	12	1370C→T	Heterozygous	P457L
4 + 8	7	718-724Δ	Heterozygous	Del G + C
	21	2728C→T	Heterozygous	R910Stop
5	19	2279delG	Homozygous	Frameshift stop AA776
6 + 9	29	4143-4144InsA	Homozygous	Frameshift stop AA1386

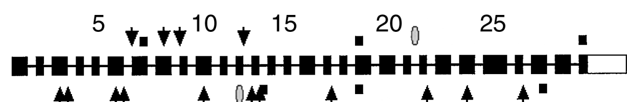

Fig. 2. Mutations identified in the *ADAMTS13* gene. Mutations of the present communication are depicted above the corresponding exons. Mutations previously described in literature are displayed below the corresponding exons. Symbols are: (▲) missense mutations; (■) deletions/insertions and splice-site mutations; (○) nonsense mutations.

DISCUSSION

The hypothesis that vWF-cleaving protease deficiency is central to the pathogenesis of familial TTP has recently been supported by the identification of several mutations in the *ADAMTS13* gene in patients with familial TTP [11, 15]. In the present study, the specific involvement of this protease in the etiology of this disorder is further substantiated by the finding of eight novel mutations in a cohort of six unrelated TTP families from different geographic origins.

Clinical data of the six patients and three affected siblings revealed important information. The diagnosis of TTP was made between 4 and 64 months of age. Seven had, however, already symptoms in the neonatal period. Interpretation of clinical data in patient 1 is complicated by an associated streptococcal septicemia during the neonatal period. In the neonate vWF-cleaving protease level is decreased (average 52%; range, 25% to 118%) [16], but still in the range allowing cleavage of the large multimers of vWF [10]. UlvWF multimers are present in the majority of the plasma of the fetus and full-term and preterm neonates. These UlvWF multimers disappear within a few weeks after birth [17]. UlvWF multimers, which are under normal circumstances not present in children and adults, contribute to platelet aggregation and hemostasis in the normal fetus and neonate. Plasma vWF in neonates is more multimerized than in adults and this explains the increased platelet deposition on subendothelium under flow conditions [18]. The possibility exists that in patients with a congenital form of TTP, there are even more UlvWF multimers in the fetal and neonatal phase than in normal fetuses and neonates. Possibly these UlvWF multimers cause thrombi and are thus responsible for the first signs of TTP. The manifestation of TTP in our patients after a certain honeymoon period suggests a physiologic maturation of the platelet function. The follow-up of our patients is reassuring, even when no prophylactic plasma infusions were given. This is certainly an unexpected finding. Only one patient had a decrease of GFR with proteinuria and microscopic haematuria. One patient showed epileptic seizures and one Legg-Calves-Perthes of the right hip. Legg-Calves-Perthes disease can be the consequence of thrombotic events [19].

Among the *ADAMTS13* mutations identified in our TTP cohort, three are likely to have a deleterious effect on the function of ADAMTS13. The nucleotide deletion (patient 5), the nonsense mutation (patients 4 and 8) and the insertion (patients 6 and 9) will cause premature termination of translation and result in truncated ADAMTS13 proteins. Half of the mutations detected in our familial TTP cohort, however, are amino acid substitutions. Our data together with the results of previous studies [11, 15] indicate that 15 out of a total of 23 identified mutant *ADAMTS13* alleles are missense mutations. These mutations are inferred to be pathogenic when they substitute highly conserved amino acids, which in view of their conservation through evolution are presumed to be of functional importance. All four missense mutations detected in our TTP cohort are substitutions of strongly conserved into nonconservative amino acids and were not found in 50 control chromosomes. Therefore, it is likely that the majority of these missense mutations are indeed harmful mutations and not innocuous polymorphisms. However, to prove that these amino acid substitutions can indeed result in impairment or loss of function will require study of the effect of individual mutations on vWF cleavage in a functional expression system [15]. By expression analysis in HeLa cells Kokame et al [15] could demonstrate that vWF-cleaving protease containing two specified mutations were not secreted from cells, while in the case of two other mutations, mutants were normally secreted but showed minimal activity. These findings suggest that the clinical symptoms of the patients will be heterogeneous.

The eight mutations detected in our study were present in TTP patients from different geographic regions. This indicates that a common ancestor is unlikely.

Of note, we were unable to identify a mutant allele in another patient, despite documented vWF-cleaving protease deficiency. This patient has a typical history of

congenital TTP, presenting at birth with jaundice and has required prophylactic infusions with fresh-frozen plasma to prevent symptomatic episodes. Although inhibitory antibodies against vWF-cleaving protease can be present already in childhood [20], these inhibitors were absent in this child. Several explanations can be offered. First, mutations may be present in gene-regulating fragments such as promoter or enhancer segments, intron sequences or 5′ and 3′ noncoding regions, which have not yet been screened for mutations. Second, large heterozygous *ADAMTS13* gene deletions will not be identified by mutation detection techniques based on analysis of individual exons. A defect in thrombospondin-1, acting as disulfide bond reductase, can be another but still undetected cause for TTP in children [21].

In view of the observation that mutations in the *ADAMTS13* gene have been identified in a high percentage of familial TTP patients studied to date, we conclude that the analysis of the *ADAMTS13* gene may be regarded as a powerful diagnostic aid. Accurate diagnosis of congenital TTP is important as symptomatic episodes can be prevented by periodic plasma transfusions. The use of humanized monoclonal antibodies against the extracellular domain of glycoprotein 1b (GP1b) modulating von Willebrand–mediated platelet adherence could offer another preferred form of treatment [22–24]. Repeated fresh plasma infusions are accompanied by risk of allergic reactions and transmission of undetected viruses.

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