The first deletion mutation in the TSP1-6 repeat domain of ADAMTS13 in a family with inherited thrombotic thrombocytopenic purpura

Roberta Palla,[1] Silvia Lavoretano,[1] Rossana Lombardi,[1] Isabella Garagiola,[1] Mehran Karimi,[2] Abdolreza Afrasiabi,[2] Mani Ramzi,[2] Raimondo De Cristofaro,[3] and Flora Peyvandi[1]

[1]Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre, University of Milan, Department of Medicine and Medical Specialities, IRCCS Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Luigi Villa Foundation, Milan, Italy; [2]Hematology and Thrombosis Unit, Haematology Research Centre, Shiraz University of Medical Sciences, Shiraz, Iran; [3]Haemostasis Research Centre, Institute of Internal Medicine and Geriatrics, Catholic University School of Medicine, Rome, Italy

ABSTRACT

The inherited deficiency of *ADAMTS13* is usually associated with severe forms of thrombotic thrombocytopenic purpura. Among the mutations identified in the *ADAMTS13* gene, none have been described on the TSP1-6 repeat domain. We investigated an Iranian family with a history of chronic recurrent thrombotic thrombocytopenic purpura, severe *ADAMTS13* deficiency and a heterogeneous pattern of clinical symptoms among affected members. Genetic analysis revealed a homozygous deletion of nucleotides 2930-2935 (GTGCCC) in exon 23 of *ADAMTS13*, leading to the replacement of Cys977 by a Trp and the deletion of Ala978 and Arg979 in the TSP1-6 repeat domain. To explore the mechanism of ADAMTS13 deficiency, *in vitro* expression studies were performed. Western blotting, pulse-chase labeling and immunofluorescence studies demonstrated a secretion pathway defect of the mutant protein, with no intracellular accumulation. This finding is consistent with the severe *ADAMTS13* deficiency but does not explain the heterogeneous clinical picture of the 3 siblings carrying the same mutation.

Key words: *ADAMTS13*, thrombotic thrombocytopenic purpura, TSP-1 repeat, deletion, mutation.

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Introduction

The identification of the von Willebrand factor-cleaving protease *ADAMTS13*[1] provided new insights into the pathophysiology of thrombotic thrombocytopenic purpura (TTP), a life threatening disorder characterized by thrombocytopenia, Coombs negative hemolytic anemia and ischemia in various organs resulting from diffuse platelet thrombi in the microcirculation. The acquired form of the disease is caused by anti-ADAMTS13 autoantibodies, whereas mutations on *ADAMTS13* gene are responsible for recessively inherited TTP. Inherited TTP usually develops during childhood, but cases with adult onset are also being reported, often triggered by events that induce release from vascular endothelial cells of ultralarge von Willebrand factor (ULVWF) multimers that are highly reactive with platelets. More than 70 *ADAMTS13* gene mutations have so far been identified in inherited TTP.[2-12] Most patients are compound heterozygotes, but a few homozygotes have also been reported, particularly in consanguineous families. The majority of the reported mutations are missense (>50%), followed by splice site, nonsense and frameshift mutations. The majority of mutations are located at the N-terminal of the protease, emphasizing the importance of these domains in VWF cleavage. *In vitro* expression studies have shown that most of the analyzed mutations determine the clinical and laboratory phenotypes through the impairment of ADAMTS13 synthesis and/or secretion.[5,6,9,11,12] Given this, we carried out a molecular investigation in a large Iranian family with a history of chronic recurrent TTP, in which both the clinically affected members presented their first episode of TTP during adulthood. DNA

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Correspondence: Flora Peyvandi, MD, PhD, Angelo Bianchi Bonomi Hemophilia and Thrombosis Centre, University of Milan, Department of Medicine and Medical Specialities, IRCCS Maggiore Hospital, Mangiagalli and Regina Elena Foundation, Luigi Villa Foundation, Via Pace 9, 20122 Milan, Italy. E-mail: flora.peyvandi@unimi.it

analysis identified a homozygous deletion of nucleotides 2930-2935 (GTGCCC) in exon 23 of *ADAMTS13* in the 2 probands, but also in one asymptomatic sibling.[13] In order to explain the patients' phenotype, we then studied the mechanistic effect of the deletion by means of expression studies in mammalian cells.

Design and Methods

Patients

Two South Iranian patients (2 brothers), off-spring of first cousins, were affected by chronic recurrent TTP that first developed during adulthood (Figure 1). Patient II:2, a 26 year old male, had his first episode of TTP at the age of 23 years and 6 subsequent recurrent episodes with no precipitating event or triggering agent. Bleeding symptoms such as purpura and petechiae were present at each episode, accompanied by fever and vomiting, whereas mild neurological symptoms (visual disorders and drowsiness) were observed only during the first episode. The clinical diagnosis was established at the time of the first episode by the presence of thrombocytopenia (platelet count no higher tha 20×10^9/L), Coombs negative hemolytic anemia (Hb 10.3 g/L), fragmented erythrocytes and high serum level of lactate dehydrogenase (LDH 1055 UI/L). The patient was successfully treated during each acute episode with plasma exchange and high dose of corticosteroids, before the molecular diagnosis. After his sixth episode of TTP, to prevent further relapses, the patient started a prophylactic treatment with fresh frozen plasma (FFP) (30 ml/kg) every three weeks. Patient II:3, a 31 year old male, developed his first episode of TTP at the age of 29 years, in association with an episode of pneumonia. He had purpura and petechiae on his legs, a platelet count of 29×10^9/L and Coombs negative hemolytic anemia (Hb 8.5 g/L, LDH 954 UI/L). Daily FFP infusions (30 mL/kg) were effective as reflected by a progressive increase in the platelet count. Since the first disease episode, the patient receives an FFP infusion when his platelet count falls below 100×10^9/L. According to the family history, another male sibling (II:1) had a TTP episode at the age of 23 years and died because of multiorgan failure. Another 4 brothers and one sister are all healthy and have never had any signs or symptoms of TTP. This study was carried out with the approval of the local ethics committee

Ex-vivo analysis

ADAMTS13 activity and antigen were measured in plasma samples and in the conditioned media of cells transfected by wild type ($ADAMTS13_{WT}$) and mutant ($ADAMTS13_{del6bp}$) expression vectors using respectively a collagen binding assay (CBA) and an immunoassay, both modified as previously described.[14] The presence of neutralizing (inhibitors) and non-neutralizing anti-ADAMTS13 autoantibodies in patients' plasma was also evaluated.[14] The coding region and intron–exon boundaries of the ADAMTS13 gene (NT_017539) were amplified and sequenced as previously reported.[5]

In vitro expression studies

The complete ADAMTS13 wild-type cDNA was inserted into the mammalian expression vector pcDNA™3.1/V5-His TOPO®TA (Invitrogen, Carlsbad, CA, USA). The deletion of 6 nucleotides from the position 2930 to 2935 of the *ADAMTS13* cDNA (NM_017587) was obtained by site-directed mutagenesis of $ADAMTS13_{WT}$ expression vector using a QuickChange™Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA, USA) using forward (5'-CGGAGGATCCTGTATTGGGCCCATGGGGAG-GACG-3') and reverse primers (5'-CGTCCTCCC-CATGGGCCCAATACAGGATCCTCCG-3'). The presence of the 6 nucleotides deletion was confirmed by sequencing analysis. To explore the functional significance of the mutations, a transient transfection assay was performed as previously described,[5] and conditioned media and lysates of cells transfected by $ADAMTS13_{WT}$ and $ADAMTS13_{del6bp}$ expression vectors were harvested.

Western blotting analysis

Samples of cell lysates and conditioned media of transiently transfected cells by $ADAMTS13_{WT}$ and $ADAMTS13_{del6bp}$ expression vectors, adjusted according to the transfection efficiency as measured by luciferase assay,[5] were analyzed by Western blotting using an anti-V5 monoclonal antibody against the C-terminal tag of rADAMTS13 (Invitrogen).[5] The amount of intracellular and extracellular rADAMTS13 proteins was quantified by densitometric analysis using a specific software program (Image Master; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunofluorescence studies

Immunofluorescence studies were performed on an African green monkey kidney, SV40 virus transformed cell line (COS-7), using an anti-V5 monoclonal antibody against ADAMTS13 and monoclonal antibodies recognizing the proteins GM130 (a cis-Golgi marker) and Bip-GRP78 (a chaperone protein involved in Golgi–endoplasmic reticulum [ER] transport) (BD Biosciences, Franklin Lakes, NJ, USA).[5]

Metabolic labeling studies

Forty-eight hours after transfection, HEK293 cells were depleted of methionine for 60 min and labeled for 60 min with 500 μCi/mL PRO-MIX™ (~73% L–[35S] methionine and ~22% L–[^{35}S] cysteine; Amersham Biosciences, Uppsala, Sweden). After a chase of 0, 3, 7 and 24 hours, performed in 1 mL of Opti-MEM I (Invitrogen), conditioned media and cell lysates were harvested adding PMSF (1 mmol/L) and precleared overnight at 4°C with 300 μL Staphylococcus aureus Cowan I (SAC) (Sigma-Aldrich, St.Louis, MO USA) coupled with a rabbit anti-mouse IgG (Sigma-Aldrich) in NP-40 lysis buffer. Recombinant proteins were immunoprecipitated adding 5 μg of anti-V5 monoclonal antibody (Invitrogen) for three hours at 4°C, followed by 100 μL of protein A Sepharose (Sigma-Aldrich) coupled 1:10 with a rabbit anti-mouse IgG (Sigma-Aldrich) in NP-40 lysis buffer. Resulting pellets were resuspended in 40 μl of PAGE sample buffer and denatured at 95°C for five minutes. The immunoprecipitated proteins were resolved on 8% SDS-PAGE. After five hours of electrophoresis the incorporated radioactivities were read on dried gels, using Imaging Analyser Typhoon 8600 (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Results and Discussion

Figure 1 shows that patients II:2 and II:3 and their asymptomatic sister (II:4) had a severe ADAMTS13 deficiency. A moderate deficiency of both activity and antigen was observed in the patients' father (I:1) whereas the mother (I:2) and one sibling (II:6) had low borderline levels of ADAMTS13 activity and antigen. The remaining family members had normal ADAMTS13 activity and antigen levels. No anti-ADAMTS13 autoantibodies (with or without neutralizing activity) were found in the patients' plasma.

Analysis of *ADAMTS13* (NT_035014) identified a deletion of 6 nucleotides GTGCCC at position 2930-2935 (c.2930_2935del GTGCCC) in exon 23, leading to the replacement of Cys977 residue by a Trp and the deletion of two aminoacids Ala978 and Arg979 (p.C977W+p.A978_R979del)[13] in the TSP1-6 repeat domain of ADAMTS13 protein. Both patients and their asymptomatic sister (II:4) were homozygotes for this mutation, their parents and 2 of their brothers (II:6 and II:7) were heterozygotes (Figure 1). Three common *ADAMTS13* intragenic SNPs p.R7W, p.Q448E and p.P618A[15] were also identified in heterozygous state in paternal alleles (I:2) and also in II:5 and II:8.

TSP1 repeats are protein modules initially identified in TSP-1, a multifunctional protein present in platelet α-granules and released upon their activation. ADAMTS13 has eight TSP1 repeats: the first is located between the disintegrin-like and the Cys-rich domains; the remaining seven are located between the spacer domain and the two C-terminal CUB domains. A few studies support the views that the seventh and eighth TSP-1 repeats and the CUB domains are dispensable for protease activity.[16] On the other hand, Majerus *et al.*[17] showed that binding of ADAMTS13 to immobilized VWF is positively modulated by the first six C-terminal TSP-1 and CUB domains. The p.C977W+p.A978_R979del mutation is located in the TSP1-6 repeat domain, where no mutations have so far been described. A Secondary Structure Prediction analysis of this mutation led us to hypothesize an alteration of the correct folding of ADAMTS13: the substitution of the residue Cys977 with a Trp probably disrupts a potential disulphide bond within the TSP1-6 domain and causes a loss of the antiparallel three-stranded fold of the TSP-1 like domains.[18] We also hypothesized that the unfolded ADAMTS13 protein might be retained in the cytoplasm causing an impaired secretion and hence the undetectable ADAMTS13 antigen and activity measured in patients' plasma.

Given this, we performed transient expression studies in HEK293 that showed a secretion failure of the mutant protein with a significantly lower concentration than rADAMTS13$_{WT}$ detected in the cell conditioned media. On Western blotting the mutant protein rADAMTS13$_{del6bp}$ appeared as a very weak band, which was estimated to be 5% of the rADAMTS13$_{WT}$, and by antigen assay rADAMTS13$_{del6bp}$ was approximately 1% (10 ηg/mL) of the rADAMTS13$_{WT}$ (870 ηg/mL). The secreted mutant protein had a normal specific activity (6% of rADAMTS13$_{WT}$).

The impairment of ADAMTS13 secretion pathway was also verified by metabolic labeling studies, showing that the mutant recombinant protein did not follow the same protein secretion pathway of the wild-type protein (Figure 2). rADAMT13$_{WT}$ in cell lysates was maximal at 0 and 3 hours of chase and started to decrease at 7 hours when the protein was completely secreted. In conditioned media, rADAMTS13$_{WT}$ was barely detectable at 3 hours and then started to accumulate reaching the maximal concentration at 24 hours. The lysates of cells expressing rADAMTS13$_{del6bp}$ showed a reduced protein amount compared to rADAMTS13$_{WT}$ at starting point and after 3 hours of chase (60% of rADAMTS13$_{WT}$), and at 7 hours of chase the rADAMTS13$_{del6bp}$ had completely disappeared. In conditioned media, rADAMTS13$_{del6bp}$ was detectable at 3 hours of chase, with a significant reduced concentration compared to rADAMTS13$_{WT}$ similar to the

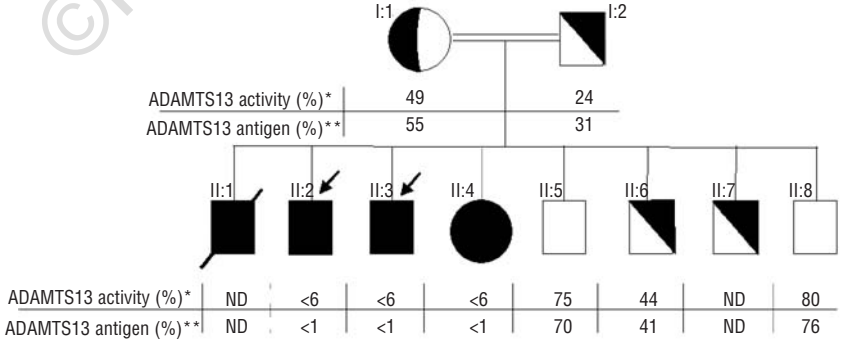

Figure 1. Pedigree of an Iranian family with congenital TTP carrying the c.2930_2935delGTGCCC mutation. Squares and circles indicate male and female, respectively, and arrows indicate the 2 siblings with symptomatic TTP. Solid circles and squares indicate the presence of the c.2930_2935delGTGCCC mutation in the homozygous state. The half-solid circles and squares indicate asymptomatic heterozygotes. Plasma levels of ADAMTS13 activity and antigen (given as percentage of pooled normal plasma), measured in the 2 patients (II:2, II:3) during the remission phase of the disease distantly from FFP infusion, and in all family members, are also shown. The plasma and DNA samples of patient II:1 were not available for phenotype and genotype analysis since the patient had died because of multiorgan failure. The plasma samples of subject II:7 were not available for phenotype analysis. ND denotes not determined. *Reference values: 46-116% **Reference values: 45-150%.

previous experiment (8% of rADAMTS13$_{WT}$). At 7 hours of chase the rADAMTS13$_{del6bp}$ was no longer detectable.

These results were confirmed by immunofluorescence studies, which showed that the mutant protein was diffusely present throughout the cytoplasm, with only a minimal amount localized at ER and cis-Golgi compartments (Figure 3A-B).The consequences of the 6 nucleotides deletion mutation highlights the importance of proper formation of disulphide bridges in ADAMTS13 function, as suggested by the identification of as many as 14 additional missense mutations involving cysteine residues in patients with inherited TTP.[2,3,6,8,9,19]

In vitro experiments were carried out in the attempt to recapitulate the effect of the 6 nucleotides deletion on the ADAMTS13 secretion pathway and on its protease activity. The mutant rADAMTS13$_{del6bp}$ protein, although minimally, was secreted and had functional activity. Hence these experiments failed to fully recapitulate the patients' plasma pattern (ADAMTS13 antigen and activity were undetectable). This discrepancy could be due to the presence of a strong CMV promoter (human cytomegalovirus immediate-early promoter) inserted in the expression vector[20,21] that may be responsible for the higher ADAMTS13 expression and activity measured in the *in vitro* experiments. The moderate reduction of ADAMTS13 activity and antigen levels observed in the patients' father (I:2) could also be explained by the presence of the p.P618A polymorphism, which was previously shown to be associated with a severe reduction of ADAMTS13 activity and antigen levels.[22] The patients' sister (II:4) remained asymptomatic until now at the age of 24 years and has not yet been pregnant. She has never developed an acute TTP

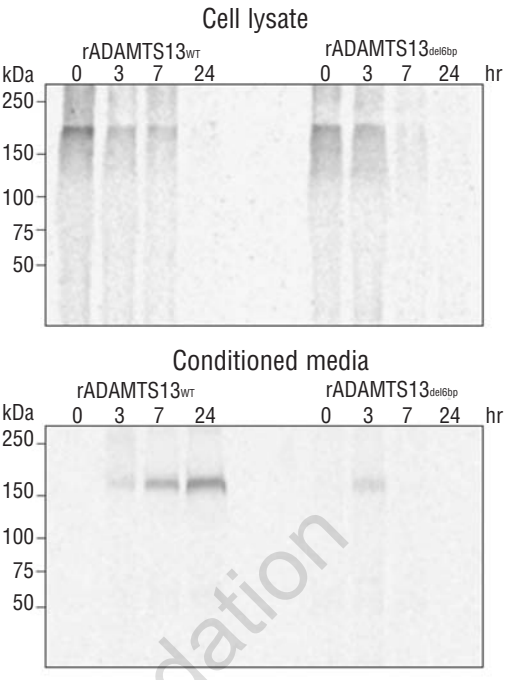

Figure 2. Pulse-chase labeling experiments in HEK293 cells transfected with ADAMTS13$_{WT}$ and ADAMTS13$_{del6p}$ expression vectors. After 60 minutes-pulse with [^{35}S] methionine, the cells were chased for 0, 3, 7 and 24 hours. Equivalent amounts of cell lysates (top) or conditioned media (bottom) for both WT and mutant constructs were immunoprecipitated using an anti-V5 monoclonal antibody against the C-terminal tag of rADAMTS13 and analyzed by 8% SDS-PAGE. The molecular weight marker is localized on the left.

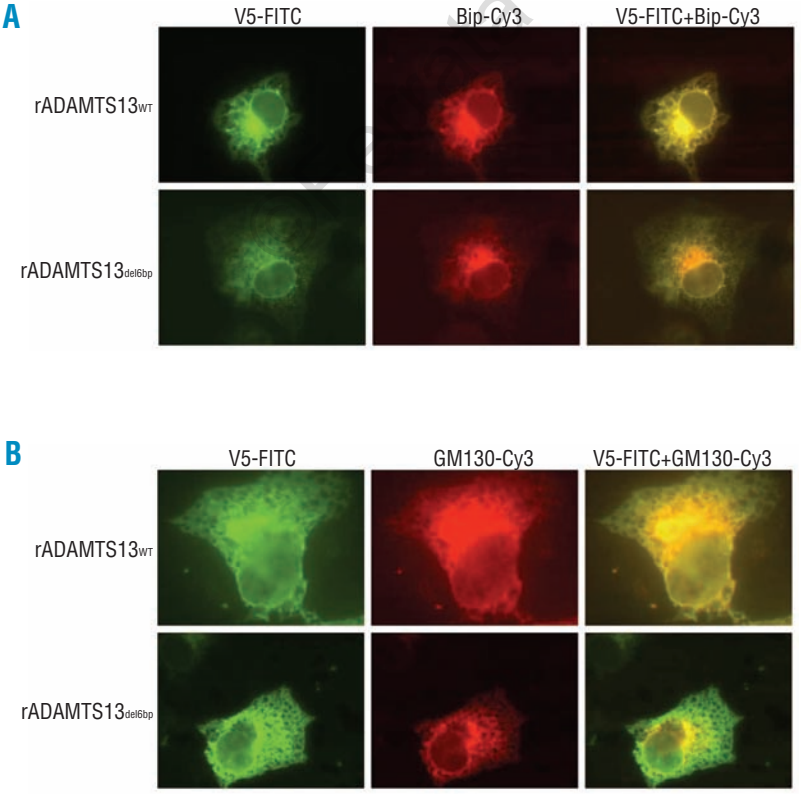

Figure 3. Dual labeling of the recombinant ADAMTS13 proteins and markers of ER and cis-Golgi compartments. (A) COS-7 cells transfected with WT and mutant expression vectors stained simultaneously with anti-V5 monoclonal antibody against rADAMTS13 (green) and anti-Bip-GRPp78 monoclonal antibody (red) against a chaperon protein of ER compartment. (B) COS-7 cells transfected with WT and mutant expression vectors stained simultaneously with anti-V5 monoclonal antibody against rADAMTS13 (green) and anti-GM130 monoclonal antibody (red) against a cis-Golgi matrix protein. The yellow colour demonstrates the colocalization of rADAMTS13$_{WT}$ in both compartments whereas cell expressing rADAMTS13$_{del6bp}$ showed a very faint dotted yellow colour in both compartments.

episode, in spite of the same homozygous genetic defect and severe ADAMTS13 deficiency found in her affected brothers (II:2, II:3). This observation, together with other previous reports,[19,23] shows once again that, in addition to severe ADAMTS13 deficiency, other as yet obscure genetic or environmental factors are required for the onset of TTP, as also shown in a murine model.[24]

In conclusion this work helped to clarify the mechanism by which the 6 nucleotides deletion mutation causes ADAMTS13 deficiency in congenital TTP. However, it corroborates the views that ADAMTS13 deficiency is necessary but not sufficient for the development of this disease, emphasizing the need to better ascertain additional factors responsible for TTP pathogenesis.

Authorship and Disclosures

RP and SL designed the study, performed the *in vitro* experiments, analyzed the results and wrote the manuscript; RL and IG performed ADAMTS13 phenotype measurement and genotype characterization; MK, AA, and MR collected DNA and plasma samples of patients and family members; RdC performed the recombinant ADAMTS13 purification and the SSP analysis; FP critically revised the study and the manuscript.

All authors approved the final version of the manuscript.

The authors report that they have no conflict of interest.

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