Nonsense-mediated mRNA decay in the *ADAMTS13* gene caused by a 29-nucleotide deletion

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ABSTRACT

Background
In mammalian cells a regulatory mechanism, known as nonsense-mediated mRNA decay, degrades mRNA harboring premature termination codons. This mechanism is intron-dependent and functions as a quality control mechanism to eliminate abnormal transcripts and modulates the levels of a variety of naturally occurring transcripts.

Design and Methods
In this study, we explored the molecular mechanism of ADAMTS13 deficiency in two compound heterozygous siblings carrying a 29-nucleotide deletion mutation located in exon 3 (c.291_319delGGAGGACACAGAGCGCTATGTGCTCACCA) in one allele and a single base (A) insertion mutation (c.4143_4144insA) in the second CUB domain previously reported in the other allele. Real-time quantitative reverse transcriptase polymerase chain reaction was used to explore whether the premature termination codons introduced by the deletion of the 29 nucleotides triggered the nonsense-mediated mRNA decay.

Results
In vitro-expression studies demonstrated that the premature termination codons inserted by the 29 bp deletion probably lead to a reduction of ADAMTS13 mRNA levels through the regulatory mechanisms of nonsense-mRNA decay. Furthermore, the 4143_4144insA mutation causes an impairment of secretion that leads to retention of the mutant protein in the endoplasmic reticulum, as observed in immunofluorescence studies.

Conclusions
In conclusion, this work reports how two different *ADAMTS13* gene defects acting at two different levels, i.e, impairment of steady-state mRNA level caused by the premature termination codon mediated decay mechanism induced by the 29 bp deletion mutation and alteration of the secretion pathway due to 4143_4144insA, lead to a severe deficiency of ADAMTS13.

Key words: ADAMTS13, *in vitro* expression study, mRNA expression, nonsense-mRNA decay.

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The online version of this article contains a supplementary appendix.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a severe microangiopathy typically characterized by thrombocytopenia, mechanical hemolytic anemia, neurological and renal manifestations, and fever.[1] TTP is associated with a deficiency of von Willebrand factor – cleaving protease and with an increase of uncleaved von Willebrand factor of ultralarge molecular weight.[2-4] The protease, designated ADAMTS13 because of its characteristic combination of a disintegrin-like and metalloprotease with thrombospondin type 1 (TSP1) motif, cleaves the platelet adhesive protein von Willebrand factor at the peptide bond Tyr1605 and Met1606.[2,5-7] The human *ADAMTS13* gene maps to chromosome 9q34 by genome-wide linkage analysis,[8] spans 37 kb and comprises 29 exons that encode a polypeptide of 1427 amino acid residues.[9,10] ADAMTS13 deficiency may be either congenital, due to mutations in *ADAMTS13*, or acquired due to neutralizing or non-neutralizing autoantibodies.[11-13] Congenital TTP is a rare disorder with undetectable or severely reduced plasma levels of ADAMTS13 as a consequence of mutations in the corresponding gene. To date, more than 80 different mutations have been identified, including missense, nonsense, and splice site alterations as well as nucleotide deletions and insertions spread across *ADAMTS13*.[14] Until now only 30% of the reported mutations have been characterized and analyzed for their consequences on the biosynthesis, secretion and activity of the protease using *in vitro*-expression studies.[15-19] The present study evaluates the molecular mechanism of two mutations observed in the compound heterozygous state in two Turkish siblings with congenital TTP. One mutation, present on the maternal allele, is a single base (A) insertion mutation located within exon 29 (c.4143_4144insA) in the second CUB domain, leading to a frameshift and loss of the last 49 amino acids of the protein.[20] The other mutation, located on the paternal allele, is a 29-nucleotide deletion mutation located in exon 3 at codon 291 (c.291_319delGGAGGACACAGAGCGCTATGTGCTCACCA) which causes premature termination codons.[21] The main goal of this study was to demonstrate how the premature termination codon introduced by the 29 bp deletion leads to a reduction of ADAMTS13 mRNA levels through such regulatory mechanisms as nonsense-mRNA decay.

Design and Methods

Patients

A Turkish male patient was referred to the Department of Pediatric Hematology of Izmir University at the age of 15 years because of a gastrointestinal infection in association with abdominal pain, fever and vomiting. Five years later, he was admitted to the hospital with purpura, renal failure and decreased platelet counts. Laboratory data on admission were as follows: Coombs-negative hemolytic anemia with schistocytes in the blood smear, hemoglobin 11.7 g/dL, low platelet count (11×10^9/L, high serum levels of lactate dehydrogenase (1809 UI/L), total bilirubin 2.6 mg/dL and creatinine 4.6 mg/dL. Laboratory results and the clinical symptoms confirmed the diagnosis of TTP. Six additional episodes occurred, usually in association with triggers such as infections or alcohol consumption. He was successfully treated with fresh-frozen plasma (10 mL/kg) and now receives prophylaxis with one infusion every 3 weeks. The patient's sister developed mild thrombocytopenia and hemolytic anemia at the age of 22 years without an acute episode of TTP up to now.

Measurement of ADAMTS13 activity, ADAMTS13 antigen and anti-ADAMTS13 antibody

ADAMTS13 activity was measured in plasma samples and in the conditioned media of cells transfected by wild type (WT) and mutant expression vectors using the collagen binding assay previously described by Gerritsen *et al*.[22] The lower limit of sensitivity was 6% of ADAMTS13 activity levels in pooled normal plasma taken as the reference standard. ADAMTS13 antigen levels were measured in plasma samples and conditioned media of cells transfected by WT and mutant expression vectors using an enzyme-linked immunosorbent assay previously described by Feys *et al*.[23,24] The presence of anti-ADAMTS13 antibodies was evaluated by western blotting analysis as reported by Peyvandi *et al*.[25] The presence of antibodies neutralizing ADAMTS13 activity was determined as previously described.[21]

Genomic sequence analysis

Genomic DNA was isolated from peripheral blood leukocytes.[26] The coding regions and intron/exon boundaries of the *ADAMTS13* gene (NT_035014) were amplified by polymerase chain reaction (PCR) and sequenced using an automated ABI PRISM™ 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Details on primers and PCR conditions are available on request. The haplotype was determined using 17 intragenic ADAMTS13 single nucleotide polymorphisms.[27]

Expression vectors

The complete *ADAMTS13* cDNA (kindly provided by Dr. F. Scheiflinger, Baxter Bioscience, Vienna, Austria) was inserted into the mammalian expression vector pcDNA™3.1/V5-His TOPO®TA (Invitrogen, Carlsbad, CA, USA). A further V5 epitope tag was inserted at the N-terminal, next to the prepropeptide of the *ADAMTS13* cDNA.

Construction of the ADAMTS13-insA expression vector

The insertion of the adenine (A) at position 4143 of the *ADAMTS13* cDNA (NM_139027) was achieved by site-directed mutagenesis of WT expression vector using a QuickChange™Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) by a forward (5'CTCTACTGGGAGTCAAGAGAGCAGCCAGGC3') and a reverse primer (5'GCCTGGCTGCTCTCTTGACTCCCAGTAGAG3'). The presence of the insertion mutation at position 4143 was confirmed by sequence analysis.

Construction and cloning of the ADAMTS13-29del expression vector

The deletion of 29 nucleotides identified in the exon 3 was inserted into the *ADAMTS13* cDNA by overlapping PCR: details are provided in an *Online Supplementary Appendix 1*).

Cell culture and transfection

Human embryo kidney (HEK) 293 cells were maintained in DMEM/F12 (1:1) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin), and glutamine (10%) at 37°C in 5% CO_2. Subconfluent HEK293 cells grown in 100-mm culture dishes were transiently transfected with 50 μg of each expression vector using electroporation according to the manufacturer's instructions (EQUIBIO/Easyject Plus; Thermo Electron Corp, Needham Heights, MA, USA). To normalize the transfection efficiency across a range of individual transfections, the reporter plasmid pRL-TK vector (Promega, Madison, WI, USA) was co-transfected as an internal reference (10:1 molar ratio of test plasmid and pRL-TK). The medium was replaced by Opti-MEM I reduced serum media (Invitrogen, Carlsbad, CA, USA) 24 h after transfection, and cells were incubated for an additional 72 h. Conditioned media of cells transfected by ADAMTS13-WT, ADAMTS13-insA and ADAMTS13-29del expression vectors were collected separately and a protease inhibitor (10% phenylmethylsulfonyl fluoride) was added, clarified by centrifugation and concentrated 30-fold using an AMICON Centricons Column (Millipore, Bedford, MA, USA). Adherent cells were washed with phosphate-buffered saline at pH 7.2 and subsequently lysed with 1 mL of 1x *Renilla* Luciferase Assay Lysis Buffer (*Renilla* Luciferase Assay System-Promega, Madison, WI, USA). Untransfected HEK293 cells were used as a negative control.

Western blot analysis

Equivalent volumes of cell lysates and conditioned media of cells transiently transfected by ADAMTS13-WT and ADAMTS13-insA expression vectors, adjusted according to the results of the luciferase assay, were resolved by 7% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The ADAMTS13-29del recombinant protein was resolved on a 15% polyacrylamide gel in order to keep its low molecular weight in view (6767 Da). WT and mutant recombinant ADAMTS13 proteins transferred to a pure nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) were detected using an anti-V5 monoclonal antibody against the N-terminal tag of recombinant ADAMTS13 (Invitrogen, Carlsbad, CA, USA) and visualized with peroxidase-labeled anti-mouse immunoglobulin G (Amersham Biosciences, Uppsala, Sweden). Electrochemoluminesce detection reagents (Amersham Biosciences, Uppsala, Sweden) followed by exposure on autoradiographic film were used for the detection.

Immunofluorescence studies

The African green monkey kidney, SV40 virus transformed cell line COS-7 was used. Immunofluorescence experiments were performed as previously reported.[17] To detect the cellular localization of WT and mutant ADAMTS13 recombinant proteins, transfected cells were stained simultaneously with anti-V5 antibody and mouse monoclonal antibodies against the protein Bip-GRP78 (a chaperone protein involved in Golgi-endoplasmic reticulum transport) (BD Biosciences, Franklin Lakes, NJ, USA). Images were captured using a Leica DMR epifluorescence microscope (Leica Imaging System, Cambridge, UK) equipped with a CCD camera (Cohu, San Diego, CA, USA) and a specific filter. The images were recorded using QFISH software (Leica Imaging System, Cambridge, UK).

Mini-gene expression vectors

An overlapping PCR technique, as described above with slight modifications, was used to insert exons 4, 5 and 6 including introns 4 and 5 into the *ADAMTS13* cDNA in frame with the tag. Two ADAMTS13 mini-gene expression vectors were constructed, one contained the WT exonic and intronic sequences and the other including the 29 bp deletion mutation. The oligonucleotides and PCR conditions are available on request.

mRNA analysis

Subconfluent HEK293 cells grown in 100-mm dishes were transiently transfected with 50 μg of each ADAMTS13-WT and ADAMTS13-29del mini-gene expression vectors. The medium was replaced by Opti-MEM I reduced Serum Media (Invitrogen, Carlsbad, CA, USA) 24 h after transfection, and cells were incubated for an additional 72 h. Cells were washed twice with phosphate-buffered saline, and total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Milan, Italy). To ensure complete removal of DNA contamination, DNase digestion was performed according to the manufacturer's recommendations. RT-PCR was performed with specific primers spanning from exon 2 to 6 of *ADAMTS13* cDNA using an Access RT-PCR System (Promega, Madison, WI, USA).

Real-time RT-PCR

Total RNA of cells transfected with ADAMTS13-WT and ADAMTS13-29del expression vectors with and without intronic regions was isolated using an RNeasy Mini Kit (QIAGEN, Milan, Italy). Primers specific to exons 5 and 6 were used for the analysis: forward 5_-GCTGACCTGGTCCTCTATATCAC-3_, reverse: 5_-AATGGTGACTCCCAGGTCGA-3_. The reference gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and was amplified using GAPDH-forward: 5'-AAAGTGGATATTGTTGCCATCA-3', and GAPDH-reverse: 5'-GGTGGAATCATATTGGAACATG-3'. Chromo4™ Detector was used as the detection system (MJ Research, Waltham, MA, USA). The results were analyzed using the previously described ΔCt comparative method.[28]

Results

ADAMTS13 antigen and activity

Table 1 reports the ADAMTS13 antigen and activity levels of both siblings and their parents. The siblings had severe ADAMTS13 deficiency, while their parents had moderately reduced plasma levels of ADAMTS13 antigen and activity. No anti-ADAMTS13 antibodies were detectable in the patients' plasma.

Genomic sequence analysis

Sequence analysis of the *ADAMTS13* gene identified two genetic defects in the heterozygous state in the siblings. The first mutation was a deletion of 29 nucleotides located at exon 3 (c.291_319delGGAGGA CACAGAGCGCTATGTGCTCACCA) causing a frameshift with premature termination codons in the metalloprotease domain.[21] The second mutation was an insertion of an adenine (A) at 4143_4144 codon in the second CUB domain, which introduced a premature termination at codon 1381 causing the loss of the last 49 amino acids at the C-terminus of ADAMTS13.[20] Table 1 reports the *ADAMTS13* gene mutations observed in both patients and their parents.

Haplotype analysis showed that both probands were carriers of the same haplotype linked to the 4143-4144insA mutation, previously reported by Schneppenheim.[27]

ADAMTS13 activity and antigen in conditioned media

ADAMTS13 antigen levels in conditioned media of cells transiently transfected by ADAMTS13-insA and ADAMTS13-29del were 3.7±1.6% and undetectable, respectively, in comparison with the level of ADAMTS13-WT taken as 100% (Table 2). The reduced amounts of ADAMTS13-insA released into the conditioned media showed an ADAMTS13 activity of 10±3.7%, compared to the ADAMTS13-WT level (the mean value of ADAMTS13-WT was set as 100%) (Table 2).

Western blot analysis

Western blot analysis performed on conditioned media and lysates of cells transfected with the ADAMTS13-WT expression vector showed a band with a molecular weight of approximately 190 kDa (Figure 1). The band was not detectable in the medium of untransfected cells used as a negative control. The lysate of cells transfected by ADAMTS13-insA expression vector showed a band with a lower molecular weight than that of ADAMTS13-WT, which reflects the loss of the last 49 amino acids at the C-terminus of ADAMTS13. Interestingly, no band was observed in the conditioned media of cells transfected by the ADAMTS13-insA expression vector, suggesting the retention of the ADAMTS13-insA recombinant protein (Figure 1A). The ADAMTS13-29del recombinant protein was not detectable in the conditioned media and cell lysates, even when a higher concentration of polyacrylamide gel (15%) was used (Figure 1B).

Immunofluorescence studies

Different patterns of localization of recombinant ADAMTS13-WT and ADAMTS13-insA were observed. ADAMTS13-WT was mainly localized in the perinuclear area (Figure 2). In contrast, ADAMTS13-insA was diffusely present throughout the cytoplasm with no perinuclear enhancement, probably consistent with a subcellular localization in the endoplasmic reticulum (Figure 2). Merge fluorescent studies demonstrated the co-localization of ADAMTS13-insA recombinant protein with a BiP-endoplasmic reticulum marker confirming the hypothesis of the retention of recombinant pro-

Table 2. ADAMTS13 activity (CBA) and antigen levels in the conditioned media of cells transiently transfected by ADAMTS13-WT, ADAMTS13-insA and ADAMTS13-29del expression vectors.

	ADAMTS13 activity (CBA)	*ADAMTS13 antigen*
r.ADAMTS13-WT	100%	100%
r.ADAMTS13insA	10 % ±3.7%	3.7% ±1.6%
r.ADAMTS13-29del	<6%	0%
HEK293 untransfected	<6%	0%

Both ADAMTS-13 activity and antigen results are expressed as a percentage of ADAMTS13-WT (mean ±SE) obtained by four different transient transfections using each single mutant and WT vector.

Table 1. ADAMTS13 phenotypes and genotypes of the two Turkish siblings affected by congenital thrombotic thrombocytopenic purpura and their parents.

	ADAMTS13 activity	*ADAMTS13 antigen*	*Muations*	
			cDNA	*Protein*
Father	55%	50%	c.291_319delGGAGGACACAGAGCGCTATGTGCTCACCA - c.4143_4144insA	p.Q97fs31X - p.S1381fs6X
Mother	67%	59%		
Patient 1 (male)	<6%	<1%	c.4143_4144insA c.291_319delGGAGGACACAGAGCGCTATGTGCTCACCA	p.S1381fs6X p.Q97fs31X
Patient 2 (female)	<6%	<1%	c.4143_4144insA c.291_319delGGAGGACACAGAGCGCTATGTGCTCACCA	p.S1381fs6X p.Q97fs31X

tein in the endoplasmic reticulum (Figure 3). For cells transfected with ADAMTS13-29del expression vector, immunofluorescence studies revealed that the mutant recombinant protein stained much more faintly than ADAMTS13-WT (Figure 2).

mRNA analysis

Since the 29 bp deletion mutation causes a frameshift in the reading frame introducing premature termination codons, reverse transcription PCR (RT-PCR) was performed to evaluate whether this mutation affects the ADAMTS13 mRNA splicing process. Total RNA extracted from HEK293 cells transiently transfected by ADAMTS13-WT and ADAMTS13-29del mini-genes were used as templates for the RT-PCR using specific primers from exon 2 to 6 of *ADAMTS13* cDNA. The RT-PCR products of ADAMTS13-WT and mutant mRNA showed two bands of 358 bp and 329 bp, respectively (Figure 4). No aberrantly spliced products were observed for mutant ADAMTS13 mRNA confirming that the 29 bp deletion mutation shows normal splicing as also demonstrated by sequencing.

Real-time RT-PCR

In order to analyze whether the premature termination codon introduced by the 29 bp deletion mutation interferes with ADAMTS13 mRNA expression levels, real-time RT-PCR studies were performed on total RNA isolated from HEK293 cells transiently transfected by ADAMTS13-WT and ADAMTS13-29del mini-genes. It was observed that the levels of expression of ADAMTS13-29del mRNA were reduced by approximately 70% in comparison with those of ADAMTS13-WT. These findings indicate that the ADAMTS13-29del mRNA bearing the premature termination codon was most likely undergoing nonsense-mediated mRNA decay (Figure 5). In order to elucidate the role played by introns in the nonsense-mediated decay mechanism, kinetics of different ADAMTS13 mRNA were evaluated in a transient transfection system using ADAMTS13-WT and ADAMTS13-29del expression vectors with no introns. This experiment showed a decrease of only 15% of steady state ADAMTS13-29del mRNA compared to ADAMTS13-WT (Figure 5) indicating that the premature termination codon introduced by the 29del mutation associated with the introns negatively affected the steady state of ADAMTS13 mRNA levels, perhaps triggering the nonsense-mRNA decay mechanisms.

Discussion

We report the identification of two mutations in a heterozygous state causing a severe deficiency of ADAMTS13 in two Turkish siblings: a 29-nucleotide

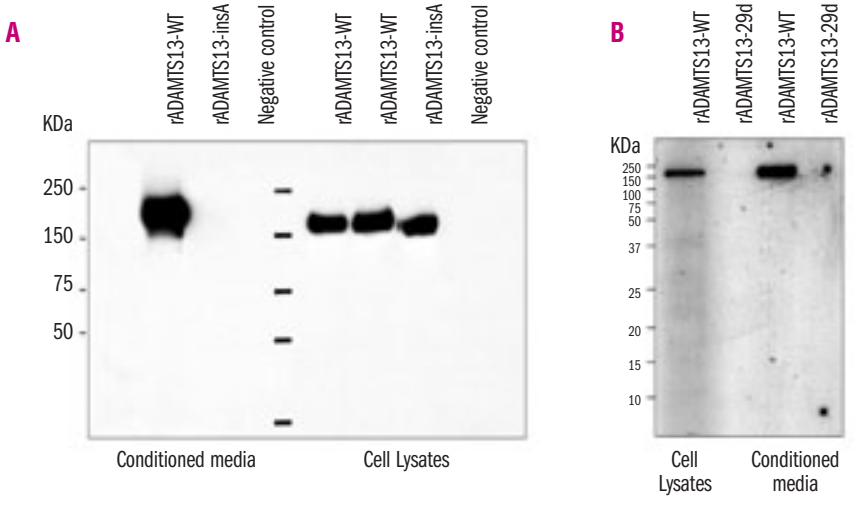


Figure 1. Western blot analysis of recombinant ADAMTS13 proteins. The ADAMTS13-WT and mutant expression vectors were transiently expressed in HEK293 cells. (A) ADAMTS13-WT and ADAMTS13-insA recombinant proteins in the conditioned media and cell lysates on 7% SDS-PAGE. (B) ADAMTS13-WT and ADAMTS13-29del recombinant proteins in the conditioned media and cell lysates on 15% SDS-PAGE. WT and mutant ADAMTS13 recombinant proteins were detected using an anti-V5 monoclonal antibody against the N-terminal tag of recombinant proteins. Negative controls were HEK293 untransfected cells.

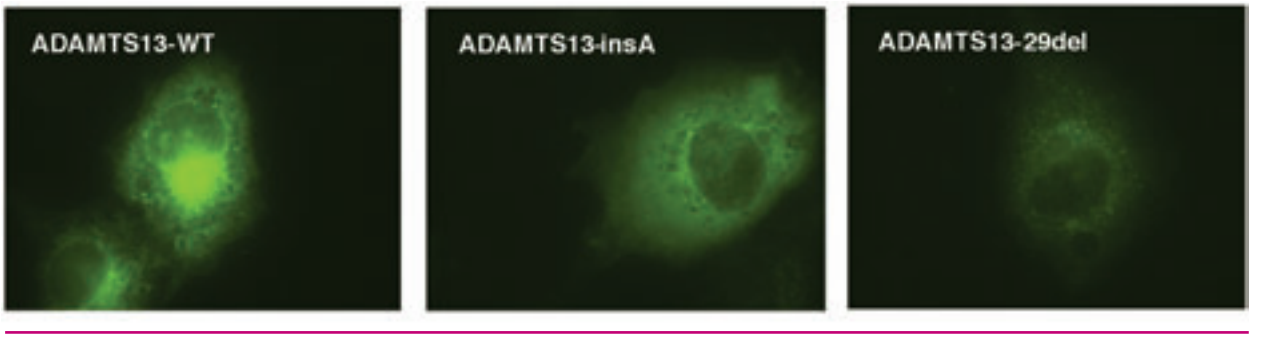


Figure 2. Immunofluorescence studies of the recombinant ADAMTS13 proteins in COS-7 cells.

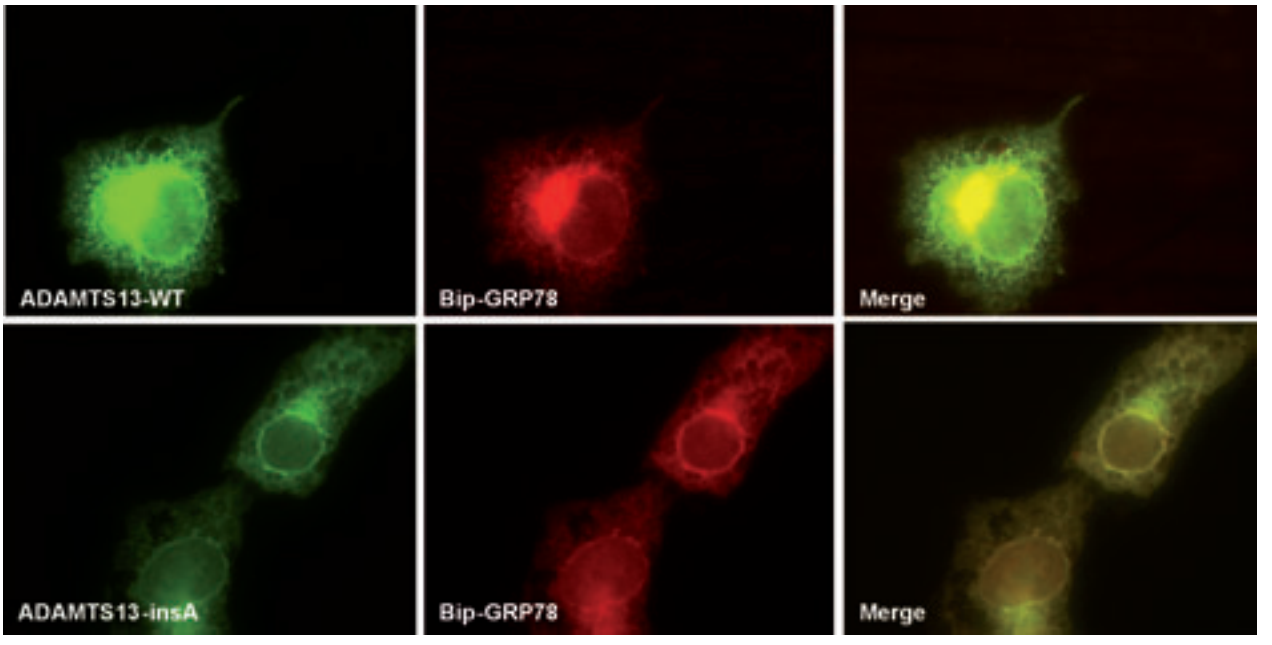
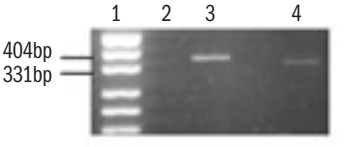


Figure 3. Merge immunofluorescence studies of ADAMTS13-WT and ADAMTS13-insA recombinant proteins. COS-7 cells transfected with WT and mutant constructs stained simultaneously with anti-V5 monoclonal antibody against recombinant ADAMTS13 (green) and anti-Bip-GRPp78 monoclonal antibody (red) against a chaperone protein of the endoplasmic reticulum compartment.

deletion mutation located in exon 3 (c.291_319del GGAGGACACAGAGCGCTATGTGCTCACCA) and a single base (A) insertion mutation located in exon 29 (c.4143_4144insA) in the second CUB domain.[20,21] With regards to the 29bp deletion mutation, we evaluated whether a regulatory mechanism, known as nonsense-mediated mRNA decay, could have any role in the level of expression of ADAMTS13 mRNA.

As reported in mammalian cells, nonsense-mediated mRNA decay is an intron-dependent biological mechanism responsible for depleting mRNA containing premature termination codons, presumably to control the synthesis of abnormal proteins deleterious to cellular metabolism.[29-31] Not all premature termination codon - bearing mRNA derived from genes containing introns are unstable. They lose stability only when the premature termination codon is located at 5' of the last intron by about 55 or more nucleotides.[32-34] We hypothesized that the premature termination codon introduced by the 29 bp deletion mutation would lead to unstable ADAMTS13 mRNA triggering the destruction of the premature termination codon bearing ADAMTS13 mRNA. First we demonstrated that the 29bp deletion mutation does not affect the ADAMTS13 mRNA splicing process. We subsequently evaluated the levels of expression, using ADAMTS13-WT and mutant minigenes, by a real-time RT-PCR technique. The expression of ADAMTS13-29del mRNA was approximately 70% lower than that of ADAMTS13-WT, indicating that the 29 bp deletion mutation negatively affects the steady state of mRNA levels.

To confirm that the nonsense-mediated decay mechanism is intron dependent, kinetic studies of ADAMTS13 mRNA using ADAMTS13-WT and ADAMTS13-29del expression vectors without introns



Lane 1 – Molecular marker

Lane 2 – Negative control

Lane 3 – ADAMTS13-WT minigene

Lane 4 – ADAMTS13-29del minigene

Figure 4. RT-PCR analysis of ADAMTS13-WT and mutant minigenes. RT-PCR products on 2% agarose gel amplified from total RNA extracted from HEK293 cells transfected with ADAMTS13-WT and ADAMTS13-29del minigenes.

were carried out. These experiments showed a decrease of approximately 15% of steady state ADAMTS13-29del mRNA using expression vectors with no introns, demonstrating that the premature termination codon introduced by the 29del mutation associated with introns negatively affects the expression level of ADAMTS13 mRNA, probably triggering the nonsense-mRNA decay mechanism. To summarize, the premature termination codon introduced by the 29 bp deletion mutation triggers a decay process reducing the expression of ADAMTS13 mRNA which probably affects the level of ADAMTS13 protein.

In the *in vitro*-expression studies, the ADAMTS13-29del recombinant protein was undetectable in conditioned media and cell lysates using western blot analysis. On the other hand immunofluorescence studies revealed that the ADAMTS13-29del recombinant protein is synthesized in small amounts as a short peptide (6767 Da) which is probably not functional and easily

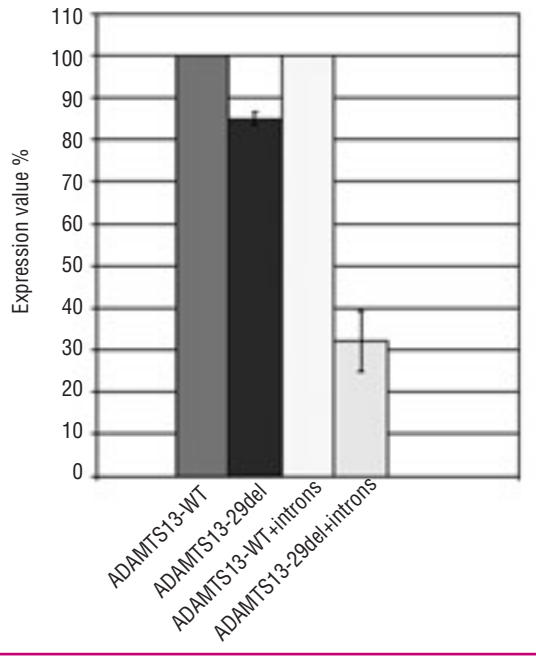


Figure 5. Real-time RT-PCR results on total RNA from ADAMTS13-WT and ADAMTS13-29del minigenes. Results of real-time RT-PCR of ADAMTS13-WT and ADAMTS13-29del without introns and ADAMTS13-WT and ADAMTS13-29del plus introns. All data are compared to ADAMTS13-WT cDNA as a calibrator sample and expressed as percentages of ADAMTS13-WT (mean±SE) obtained in three independent assays.

degradable. The lack of detection of the ADAMTS13-29del recombinant protein in the western blots could probably be explained by the recombinant protein having lost the V5-tag when the cells were lysed.

With regard to the second gene variation, the 4143_4144insA mutation located in the second CUB domain, our *in vitro* expression studies confirmed that the 4143_4144insA mutation impairs the secretion pathway associated with intracellular accumulation. The defect may be due to the removal of the central β-strands present in the CUB domain, resulting in the destruction of its architecture, as previously described by Pimanda.[18] Eight different mutations in the first and second CUB domain were reported previously and some were analyzed by *in vitro*-expression studies, suggesting, consistently with our results, that the CUB domains play a critical role in the biosynthesis and secretion of ADAMTS13.[8,35,36]

The 4143_4144insA mutation has been frequently detected in patients with hereditary ADAMTS13 deficiency in northern and central European countries. Schneppenheim and colleagues, after analyzing the segregation of 4143_4144insA mutation using 17 intragenic polymorphic markers in patients and their relatives, suggested that 4143_4144insA is a founder mutation most probably derived from a common ancestor in central Europe.[27] The identification of the ADAMTS13 haplotype linked to the 4143_4144insA mutation in our probands from Turkey is consistent with the hypothesis of a common ancestor in central Europe. This could also be due to immigration from central Europe to Turkey when, in the latter part of the 19th century, the Ottoman Empire received refugees, particularly Hungarian and Poles, from the Hasburg Empire. Furthermore Turkey also became a country of refuge for approximately 100,000 Jews from German-occupied Europe who made Turkey their country of first asylum. Hence, it is reasonable to consider that the 4143_4144insA mutation in our Turkish patients reflects a history of trading and migration between countries, which has served as a vehicle for gene flow.

In conclusion, this work demonstrates that the two cases of severe ADAMTS13 deficiency that we studied are mechanistically caused by the association of two different gene defects acting at two different levels: the impairment of steady state mRNA levels caused by a premature termination codon-mediated decay mechanism induced by a 29 bp deletion, and alteration of the secretion pathway caused by the 4143_4144insA mutation.

Authorship and Disclosures

IG, CV and SL performed the experiments; IG and FP designed the research, analyzed the results and wrote the paper. Other authors provided samples and clinical data. The authors report no potential conflicts of interest.

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