A new ADAMTS13 missense mutation (D1362V) in thrombotic thrombocytopenic purpura diagnosed during pregnancy

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Dear Sirs,

Functional deficiency of ADAMTS13 in thrombotic thrombocytopenic purpura (TTP) patients is associated with circulating ultra-large von Willebrand factor (ULVWF) multimers that display spontaneous platelet-binding capacity (1). ADAMTS13 cleaves VWF in the A2 domain of the VWF monomer (2–5).

ADAMTS13 contains 29 exons and spans 37 kb on chromosome 9q34 (6–8). It has 1,427 amino acids, 180 kDa of molecular weight and consists of a signal peptide, a propeptide, a metalloprotease domain, a disintegrin-like domain, a thrombospondin type 1 (TSP1) motif, a cysteine-rich domain, seven TSP1 repeats, and two CUB domains (6–9). Over 76 ADAMTS13 mutations have been reported (10–20). *In vitro* expression studies have shown that the most of the analysed mutations determine the clinical and laboratory phenotypes through the impairment of ADAMTS13 synthesis and/or secretion (10–14). The relevance of the CUB domains to overall function have been more heavily disputed, even though TTP-associated mutations have been found in these regions (21). The incidence of pregnancy-associated TTP is about only one in 25,000 pregnancies (22), but it accounts for approximately 10% of all TTP cases (23). Donadelli et al. (11), Camilleri et al. (13) and Fujimura et al. (24) reported mutations of the ADAMTS13 gene in pregnancy-associated TTP. Given this, we carried out a molecular investigation in a patient with a history of TTP episodes during two of three pregnancies.

A healthy 31-year-old woman was admitted to intensive care unit in the 20th week of her first gestation because of thrombocytopenia and severe anaemia with a negative Coombs' test. Packed red blood cells and prednisone were started without response. Immediate therapeutic plasma exchange (PE) using fresh frozen plasma (FFP) as replacement fluid was initiated. The patient responded to treatment, but the foetus died. She became pregnant again twice, two and four years after this crisis. FFP infusions were started immediately and sustained during pregnancies; successful caesarean sections were performed in both cases.

In the patient's plasma samples we evaluated: ADAMTS13 activity (normal values=40–130%) by chromogenic VWF-73 (chr-VWF73), IgG anti-ADAMTS13 antibodies (positive samples >15 U/ml) and ADAMTS13 antigen (normal values=70–160%) by ELISA kits (Technoclone, Vienna, Austria); VWF:Ag (normal values=53–220%) (25) and ULVWF multimers (normal value<15%) (26).

One year after the first pregnancy, the patient had <0.5% of ADAMTS13 activity, 6 U/ml IgG anti-ADAMTS13 antibodies, 2% ADAMTS13 antigen, 91 % VWF:Ag and 31% ULVWF multimers. The ADAMTS13 antigen was 2% and 119% VWF:Ag at the 24 weeks of third pregnancy, six years after the first pregnancy.

All the exons and intron-exon boundaries of the ADAMTS13 patient gene were amplified by PCR. All PCR products were directly sequenced and aligned with the ADAMTS13 gene and cDNA (7–8). DNA sequencing identified a new missense mutation A4085T in exon 29, in homozygosity state, causing the amino acid substitution of aspartic acid to valine, position 1362, in the CUB-2 domain of ADAMTS13 (D1362V). The mutation was not found in 100 alleles of healthy subjects.

Site-directed mutagenesis of WT expression vector was performed using a QuikChange® II *XL Site*-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

Subconfluent HEK293 cells were transiently transfected with each expression vector using lipofectamine. Culture media of cells transfected with ADAMTS13 WT and D1362V expression vectors were collected (48 hours after) separately and a protease inhibitor was added. Adherent cells were washed and lysed. All samples were concentrated and quantified (27). Untransfected HEK293 cells were used as a control.

The samples were subjected to SDS/PAGE (7%) under reducing and denaturing conditions, and transferred by Western

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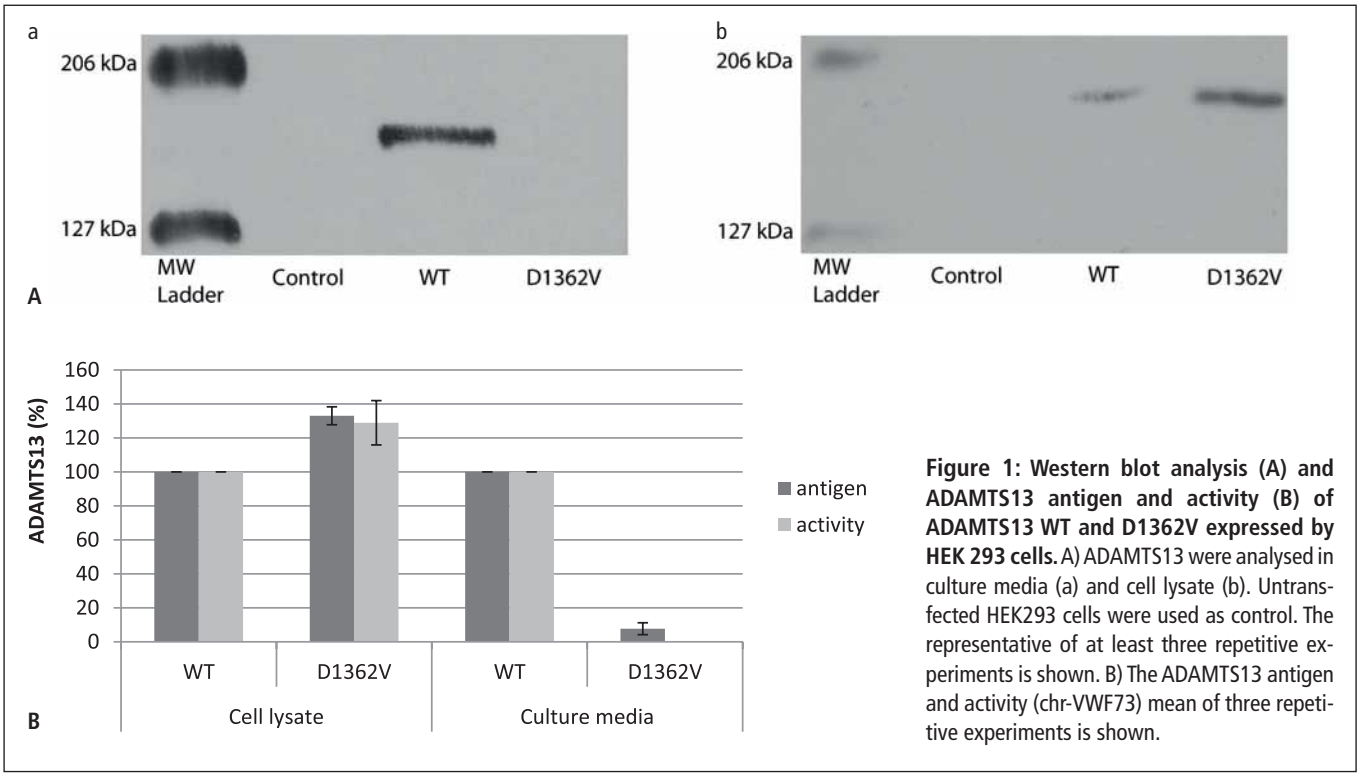

Figure 1: Western blot analysis (A) and ADAMTS13 antigen and activity (B) of ADAMTS13 WT and D1362V expressed by HEK 293 cells. A) ADAMTS13 were analysed in culture media (a) and cell lysate (b). Untransfected HEK293 cells were used as control. The representative of at least three repetitive experiments is shown. B) The ADAMTS13 antigen and activity (chr-VWF73) mean of three repetitive experiments is shown.

blot. After blocking, the membrane was incubated with anti-ADAMTS13 (ab28273–100, ABCAM; Cambridge, UK), peroxidase-labelled horse anti-goat IgG and detection was carried out by chemiluminescence. Western blot analysis performed on culture media of cells transfected with the ADAMTS13 WT expression vector showed a band with a molecular weight of approximately 190 kDa. The band was not detectable on culture media of cells transfected with the D1362V expression vector and neither in control cells, stating the lack of secretion (▶Fig. 1A, a). The lysate of cells transfected with D1362V and ADAMTS13 WT showed the same band (approximately 190 kDa), but the WT with a lower intensity than the mutant (▶Fig. 1A, b).

In culture media and cell lysates, ADAMTS13 activity and antigen were evaluated by the methods described above. The results of mutant and WT were expressed as IU/100 mg protein and were related to the WT values taken as 100%. *In vitro* expression of ADAMTS13 mutant led to a defect of secretion in culture media with ADAMTS13 antigen level of 8 ± 3% and chr-VWF73 <0.5%. This mutation produced intracellular accumulation of ADAMTS13 showing antigen level of 133 ± 5% and chr-VWF73 of 129 ± 13% (▶Fig. 1B).

All patients with the mutation c.4143–4144insA located in the CUB-2 domain encoded by exon 29 were diagnosed with severe ADAMTS13 deficiency (18). During *in vitro* experiments on the ADAMTS13 mutation, the specific activity of the mutant was low (14%) for the culture media and high (85%) for cell lysates in comparison with that of the WT enzyme (28). Antoine et al. reported (29) two brothers with severe congenital deficiency of ADAMTS13 (<3%), heterozygous for R1336W in the CUB-2 domain encoded by exon 28. The amino acid change led to a reduced secretion of the mutated protein (ADAMTS13 antigen: 23% in the conditioned medium of cells) with a significantly lower residual activity (ADAMTS 13 activity: 12%) (30). Expression studies (28, 30) indicated that the mutations in the CUB-2 domain reduce the ADAMTS13 activity but most probably not to the extent necessary to induce disease (30). We observed that the specific activity of our mutant D1362V was absent for the culture media and high for cell lysates. In our case the mutation did indeed cause ADAMTS13 deficiency *in vitro* according to the patient's ADAMTS13 activity.

We conclude that in a severe but asymptomatic ADAMTS13 deficiency in plasma, pregnancy appears to be crucial in triggering TTP symptoms. The novel mutation D1362V -identified in the CUB-2 domain encoded by exon 29– where no missense mutations have been reported yet (20), induces a plasmatic ADAMTS13 deficiency that could be caused by reduced secretion of the protease to circulation.

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Conflicts of interest
None declared.

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