revealing a three-domain architecture [5, 6]. Domain I was denoted the FYSH (fungal, Yhr087wp, Shwachman) domain and is predominantly negatively charged, whereas Domain II and III are mainly charged basic. It is predicted that Domain II and III bind to rRNA.

A majority of SDS patients have two common SBDS mutations: the first mutation in exon 2 results in premature stop codon (at c.183TA>CT, resulting in a truncated 62 amino acids) and the second a splice donor mutation in intron 2 causes a frameshift (c.258+2T>C, resulting in a truncated 84 amino acids) [3]. Both truncated proteins retain a large portion of the N-terminal Domain I. There are also two less common point mutation described, which include C31W and N34I [6]. These missense point mutations will result in a complete full-length protein with disruption of the critical FYSH domain.

Targeted disruption of *SBDS* leads to early embryonic lethality in mice [4]. We and others have reported that knockdown of SBDS protein levels with RNAi in murine hematopoietic cells results in impaired neutrophil production [7, 8]. Patients with SDS have a propensity to develop clonal cytogenetic abnormalities in the bone marrow through karyotype instability and chromosome 7 and 20 anomalies [9] and it has been shown that SBDS stabilizes the mitotic spindle to prevent genomic instability [10, 11].

SBDS protein is located on the nucleolus, where rRNA biogenesis and maturation occurs [12]. This localization has been used to support the hypothesis that SBDS functions to regulate rRNA processing, and it has been reported that SBDS associates with 28S ribosomal RNA in HeLa cells [13]. Despite a potentially important role in nuclear physiology, the *SBDS* gene does not have canonical nuclear localization signals. This has led to the speculation that nuclear localization is undertaken by a shuttling protein. Although SBDS binds to a number of proteins including nucleophosmin (NPM), Tif6 and HsNip7, none of these have been shown to promote localization to the nucleolus [13–15]. The nuclear localization mechanism of SBDS is yet unknown.

In this report, we demonstrate that the N-terminal FYSH region of SBDS protein is critical for SBDS nuclear localization.

2 Materials and methods

2.1 Generation of SBDS truncations and mutations

Full-length human SBDS gene was cloned as described previously [7]. Domains of SBDS corresponding to the 1–95aa (Domain I), 96–166aa (Domain II), 167–249aa (Domain III), Domain I–II (ΔIII), Domain I–III (ΔII) and Domain II–III (ΔI) were amplified with PCR: Domain I is the combination of 1s GAATTCTATGTCGATCTT CACCCCCAC and 1as GGATCCTCATGATACTTGAAC TTCTCCTTTAG; Domain I–II, 1s and 2as GGATCCT CATATCTTCATTTTCTCTTTTAACTGC; Domain II, 2s GAATTCTGATAAAGAAAGACACACACAAC and 2as; Domain I–III, Δ2s GAAGTTCAAGTATCAGAACGTG CTCACATGAGGC and Δ2as CATGTGAGCACGTTCT GATACTTGAACTTCTCCTT; Domain III, 3s GAATTCT GAACGTGCTCACATGAGGC and 3as GGATCCTCAT TCAAATTTCTCATCTCCTTC; Domain II–III, 2s and 3as, and cloned into pTagGFP-C vector (Evrogen, Moscow, Russia). Mutated SBDS constructs were generated by site-directed mutagenesis of pTagGFP-C-SBDS by PCR: disease-related mutations, C31W CTTCGAAATCGCCT GGTACAAAAACAAGGT, N34I TCGCCTGCTACAAA ATCAAGGTCGTCGGCTG, L71P CCAAAAAGGAA GATCCCATCAGTGCGTTTGG, K33E GAAATCGCCT GCTACGAAAACAAGGTCGTCG; conserved amino acid residue, K62E TTTGTAAATGTTTCTGAAGGTCAGGT TGCCA, E49K GGAAAAAGACCTCGATAAAGTTCT GCAGACCC, E82K ACAGATGACCAAACTAAAATCT GTAAGCAGAT, D70N GTTGCCAAAAAGGAAAAT CTCATCAGTGCGTT. These mutated SBDS genes were cloned separately into pTagGFP-C vector. Truncated SBDS constructs, K62X, GGATCCTTAAGAAACATTT ACAAACACTGAGTG, and C84X, GGATCCTCAGATT TCAGTTTGGTCATCTGTTCC, were amplified with PCR, and cloned into pTagGFP-C vector.

2.2 Cell culture and transfection

HeLa cells were cultured in Dulbecco modified Eagle medium (SIGMA), supplemented with 10% FBS. Cells were transfected with 1 μg DNA, using 1.5 μl FuGENE 6D (Roche, Mannheim, Germany) and 200 μl OPTI-MEM I medium (Gibco BRL, Gaithersburg, MD, USA) per well. After 5 h, 200 μl DMEM supplemented with 20% FBS was added and incubated for another 12 h in the presence or absence of 10 μM Lactacystin. 32Dcl3 was cultured as described previously [7]. pTagGFP-C-SBDS vector was digested with BspLU11I and transfected into 32Dcl3 by electroporation with a Gene Pulsor Xcell apparatus (Bio-Rad Laboratories, Hercules, CA, USA) at 230 V and 950 μF. Stable transfectants were selected by the addition of G418. Monoclonal cell lines were prepared with soft-agar culture.

2.3 Immunofluorescence microscopy and morphologic analyses

Anti-SBDS antiserum was prepared as described previously [7]. We further purified anti-SBDS antibody with two SBDS affinity columns. GST-SBDS column was made

with GST-SBDS immobilized glutathione Sepharose. SBDS column was made by purified recombinant non-tagged SBDS and SulfoLink immobilization kit (Pierce Biotechnology, Rockford, IL, USA). The reactivity of these antibodies to SBDS proteins was the same. HeLa cells, transfected with pTagGFP-C constructs, were washed with PBS and fixed in 4% paraformaldehyde/PBS(−) for 10 min. In the case of 32Dcl3 cells, cells were attached to poly-L-lysine coated slide grass and fixed with paraformaldehyde. After fixation, cells were permeabilized with 0.2% Triton-X100/PBS(−) for 2 min. Cells were washed 4 times with PBS(−), and then incubated overnight with affinity purified anti-SBDS antibody (1:100 dilution). Cells were then rinsed and washed 4 times with PBS(−) and then incubated with secondary antibodies coupled to fluorescein isothiocyanate (FITC), swine anti-rabbit antibodies (1:100 dilution) (DAKO, Glostrup, Denmark) or coupled to Rhodamine, donkey anti-rabbit or anti-mouse antibodies (1:200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed, and coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and sealed with nail polish. Cells were viewed using Olympus BX51 (Olympus, Tokyo, Japan), and images were acquired using ImageJ software (NIH) and processed with Photoshop (Adobe, San Jose, CA, USA). All experiments were repeated three times independently and the data were reproducible.

2.4 Western blotting

Cells were lysed with lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF, 2 μM Leupeptin and 2 μM Pepstatin A). Lysates were centrifuged at 15,000*g* for 15 min at 4°C. Nuclear and cytoplasmic extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated with 12% SDS-PAGE and transferred to Immobilon-P. SBDS and EGFP proteins were detected with both anti-SBDS antiserum and anti-pTAG antibody (Evrogen, Moscow, Russia). The results were acquired and quantified using Image J software.

2.5 Generation of Epstein–Barr virus-immortalized B cell lines

Peripheral venous blood was taken from the two SDS patients with two common mutations after informed consents were obtained. Mononuclear cells (MNC) were prepared from venous blood using Ficoll/Hypaque centrifugation method. MNC was inoculated with Epstein–Barr virus (EBV)-producing B95-8 cells in the presence of cyclosporine A [16]. EBV-transformed B-lymphoblastoid cell lines (LCL) were established in a few weeks and were maintained in RPMI1640 medium with 10% fetal calf serum (LCL-SDS1, LCL-SDS2). The human LCL cell lines (LCL-control), 277-LCL (RVB2283), was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan.

3 Results

3.1 N-terminal Domain I is responsible for nuclear localization

To ascertain which domains are responsible for SBDS nuclear localization, we generated truncated SBDS mutant constructs. These constructs were designed to delete critical domains of the SBDS protein (Fig. 1a). These truncated SBDS proteins were fused to EGFP protein and expressed in HeLa cells (Fig. 1b, c). EGFP–SBDS and EGFP-truncated SBDS proteins were detected at the expected size (Fig. 1b). EGFP–SBDS (wild type) was clearly localized to the nucleus. Proteins that had an intact Domain I, i.e., constructs EGFP–SBDS Domain I (ΔII, III), EGFP–SBDS Domain I–II (ΔIII) and EGFP–SBDS Domain I–III (ΔII), were found in the nucleus. On the other hand, deletion of Domain I, i.e., construct EGFP–SBDS Domain II–III (ΔI) and EGFP–SBDS Domain III (ΔI, II), was localized to the cytosol. Though EGFP–SBDS Domain II (ΔI, III) was found in the nucleus, EGFP–SBDS Domain II, III (ΔI) and EGFP–SBDS Domain III (ΔI, II) protein were localized to the cytosol. These results suggest that Domain II is not responsible for nuclear localization of SBDS protein and demonstrated that Domain I is critical for the nuclear localization of SBDS.

3.2 31C and 34I residues of SBDS are necessary for nuclear localization

A variety of mutation sites were reported in SDS patients since *SBDS* was identified as the syndrome responsible gene [3, 6, 9, 12, 17–28]. However, 85% of mutation sites were found in the same position, referred to as hot spots, both c.183TA>CT and c.258+2T>C. Several disease-related mutations (i.e., C31W, K33E, N34I, L71P), which were found in Domain I, did not rescue the cell growth in yeast [6]. We generated these disease-related constructs and also four mutated amino acid residues that are conserved across species (E49K, K62E, D70N, E82K) [29]. These constructs were then transfected into HeLa cells (Fig. 2a). Disease-related mutations, EGFP–SBDS C31W and EGFP–SBDS N34I, were found in the cytosol and not in the nuclei. However, EGFP–SBDS K33E and EGFP–

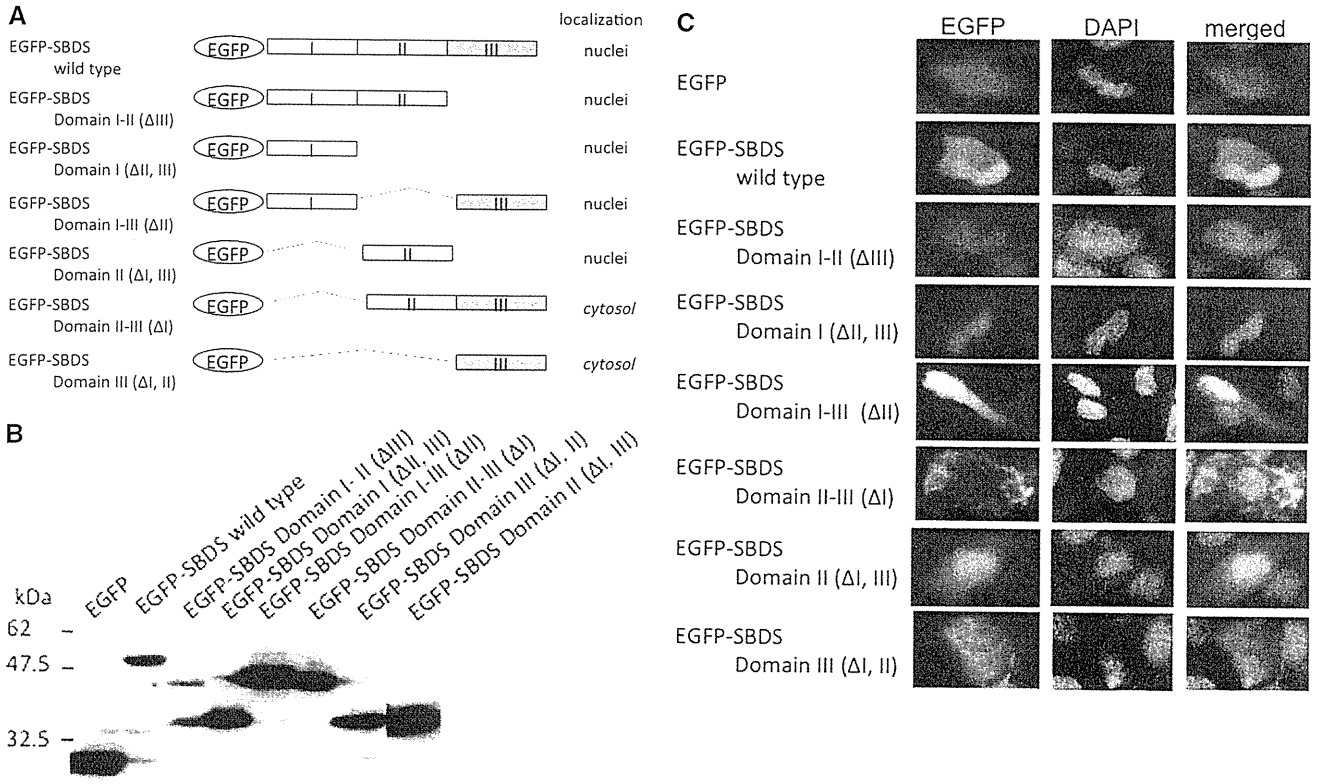


Fig. 1 N-terminal region of SBDS is necessary for nuclear localization. **a** Schematic diagram of the domain constructs. SBDS Domain I is important for nuclear localization. **b** Expression of EGFP–SBDS (wild type), EGFP–SBDS Domain I, II (ΔIII), EGFP–SBDS Domain I (ΔI, II), EGFP–SBDS Domain I, III (ΔII), EGFP–SBDS Domain II, III (ΔI), EGFP–SBDS Domain II (ΔI, III), and EGFP–SBDS Domain III (ΔI, II) in HeLa cells. Cell lysates were detected with anti-pTAG antibody. **c** Cellular localization of SBDS protein was determined. EGFP–SBDS (wild type), EGFP–SBDS Domain I, II (ΔIII), EGFP–SBDS Domain I (ΔI, II), EGFP–SBDS Domain I, III (ΔII), EGFP–SBDS Domain II, III (ΔI), EGFP–SBDS Domain II (ΔI, III) and EGFP–SBDS Domain III (ΔI, II) were transfected into HeLa cells. After 24 h, cells were fixed with 4% paraformaldehyde in PBS and counterstained with DAPI. The images are representations of at least 3 independent experiments

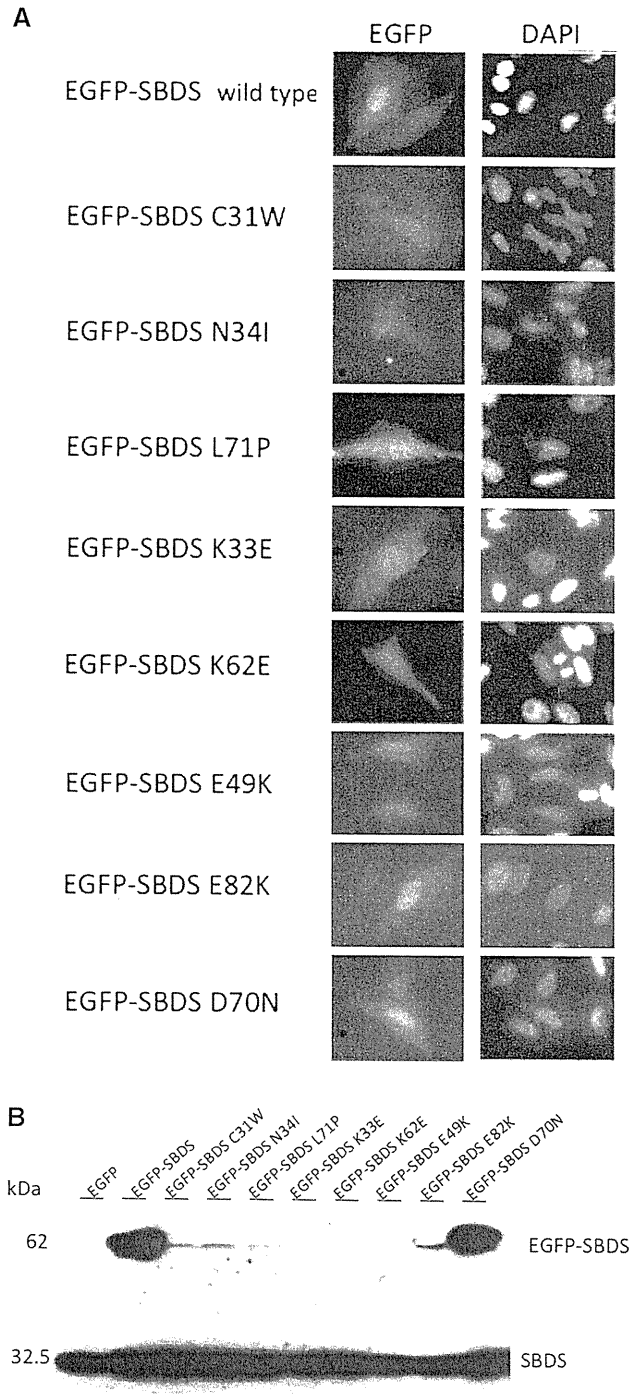
SBDS L71P were found in the nucleus. All mutations in the conserved amino acid were found in the nucleus. We further analyzed the localization of EGFP-mutated SBDS Domain I in HeLa cells. Both EGFP–SBDS Domain I C31W and -N34I were found in the cytosol and other SBDS Domain I mutants were localized to the nuclei (data not shown). These data demonstrated that 31C and 34N are critical for SBDS nuclear localization.

3.3 Mutation of the SBDS gene results in a dramatic decrease in its protein expression

The amount of mutated SBDS protein was determined with Western blotting. The amount of EGFP–SBDS (wild type) and EGFP–SBDS D70N proteins were the same. However, the amounts of all other mutated EGFP–SBDS proteins were much lower than EGFP–SBDS (wild type) (Fig. 2b). These results indicate that the stability of mutated SBDS protein was reduced. To show whether mislocalization of mutated C31W and N34I SBDS protein was a consequence of low protein amount, cells were treated with the proteasome inhibitor Lactacystin (Fig. 3). Treatment with Lactacystin resulted in an increase in SBDS protein as indicated by higher fluorescence intensity than control cells. However, EGFP–SBDS C31W and EGFP–SBDS N34I proteins were aggregated in the cytosol and not in the nuclei. These results suggest that mislocalization of EGFP–SBDS C31W and EGFP–SBDS N34I was not a consequence of low SBDS protein expression and support the conclusion that changes in amino acid residues are important for SBDS nuclear localization.

3.4 N-terminal region of SBDS polypeptide accumulates in nucleoli

We also analyzed the localization of patient's-derived truncated N-terminal peptides in HeLa cells, because almost all of Domain I is intact in these mutants. Though some degraded proteins were found, EGFP–SBDS K62X and EGFP–SBDS C84X proteins were detected at 34 and 36 kDa proteins, respectively (Fig. 4a). As expected, EGFP–SBDS K62X and EGFP–SBDS C84X were clearly



◀ **Fig. 2** N-terminal FYSH region is important for SBDS nuclear localization. **a** Cellular localization of mutated SBDS protein. EGFP-tagged SBDS (wild type), disease-related mutation (SBDS C31W, SBDS K33E, SBDS N34I, SBDS L71P) and mutation of conserved amino acids (SBDS E49K, SBDS K62E, SBDS D70N, SBDS E82K) were transfected into HeLa cells. The images are representations of at least 3 independent experiments. **b** Dramatic differences in the EGFP–SBDS protein expression level in wild-type and mutated SBDS. Protein expression level is affected by mutation of SBDS. EGFP–SBDS (wild type), disease-related mutation (SBDS C31W, SBDS K33E, SBDS N34I, SBDS L71P) and mutations of conserved amino acids (SBDS E49K, SBDS K62E, SBDS D70N, SBDS E82K) were transfected into HeLa cells. Cells were harvested after 24 h, and lysates were analyzed by immunoblotting with anti-SBDS antibody

localized to the nuclei (Fig. 4b). These results showed that 61 amino acid residues were enough for SBDS nuclear localization. To show the accumulation of N-terminal peptide in SDS patients (c.183TA>CT and c.258+2T>C), we established EBV-LCL cells from two SDS patients (LCL-SDS1 and LCL-SDS2). Figure 4c shows that a very small amount of SBDS protein was detected in LCL-SDS cells as Austin et al. [10] reported. When cells were stained with anti-SBDS antibody, strong fluorescence signals were detected in the nucleolus. Moreover, these signals disappeared in the presence of recombinant SBDS protein (Fig. 4d). Though we could not detect 6- or 8-kDa polypeptide by Western blot or immunoprecipitation by anti-SBDS antibody (data not shown), these results suggest that N-terminal immature polypeptides are accumulated in the nucleolus in SDS patients.

3.5 N-terminal region of SBDS protein competes with endogenous SBDS nuclear localization

We showed that SBDS Domain I is critical for SBDS nuclear localization. To analyze how SBDS protein accumulates in the nuclei, we established the stable EGFP, EGFP–SBDS or EGFP–SBDS Domain I overexpressed 32Dcl3 cells. Both EGFP–SBDS and EGFP–SBDS Domain I were found not only in the nuclear fraction, but also in the cytosolic fraction (Fig. 5a). Endogenous SBDS protein was found in the nuclear fraction in EGFP-expressed cells. However, the distribution of endogenous SBDS protein in EGFP–SBDS and EGFP–SBDS Domain I was changed from the nuclear fraction to the cytosolic faction. The ratios of cytosolic/nuclear SBDS protein was 0.16 ± 0.13 (EGFP), 0.83 ± 0.02 (EGFP–SBDS) and 1.41 ± 0.57 (EGFP–SBDS Domain I) (Fig. 5b). Moreover, the competition between EGFP–SBDS and endogenous SBDS was found in HeLa cells (Fig. 5c). These results suggest that EGFP–SBDS and EGFP–SBDS Domain I compete with endogenous SBDS nuclear localization.

4 Discussion

The crystal structure of the SBDS protein ortholog from *A. fulgidus* was recently determined by X-ray crystallography and it indicated a three-domain architecture [5, 6]. The SBDS protein consists of an N-terminal FYSH (fungal, YHR087wp, Shwachman) domain containing a novel mixed α/β fold, which is called Domain I. The central domain (Domain II) consists of a three-helical bundle, whereas the C-terminal domain (Domain III) has a

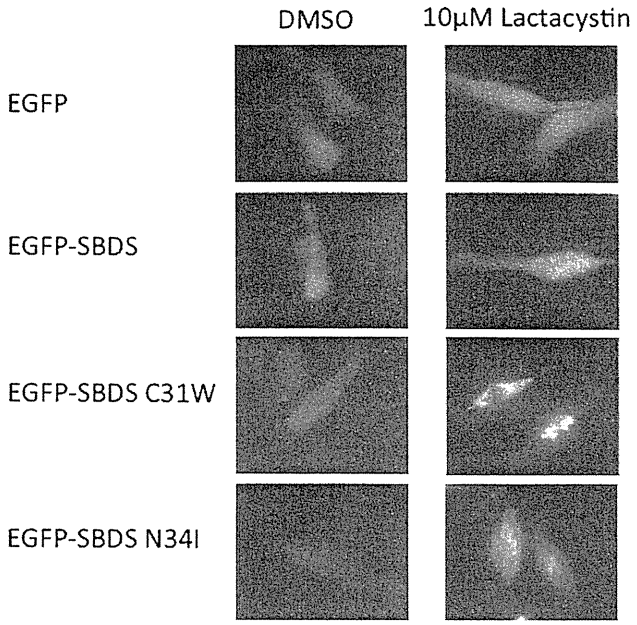


Fig. 3 Mutated SBDS proteins are degraded by proteasome. EGFP-tagged SBDS were transfected into HeLa cells in the presence or absence of 10 μM Lactacystin for 24 h. Cells were fixed with 4% paraformaldehyde in PBS

ferredoxin-like fold, which shows closest structural similarity to the Domain V of the yeast translation elongation factor 2 [5]. This observation further emphasizes the role of the *SBDS* gene in RNA metabolism. More recently, Menne et al. [14], using *S. cerevisiae* strains, have shown that the yeast ortholog Sdo1 acts with the cytoplasmic GTPase EFL1 to promote recycling of Tif6, a protein that is stably associated with pre-60-S ribosomal subunits and is required for their synthesis and nuclear export [30]. These data taken together suggest that depending on the cellular localization of the *SBDS* gene, different facets of RNA biogenesis and metabolism will be regulated. Also, Austin and Orelio [10, 11] reported that SBDS protein has a critical role in stabilizing the mitotic spindle.

In this study, we have deleted the critical domains in the *SBDS* gene and also made constructs for the common SDS-related mutations. We have used these constructs to define which domains and amino acid sequences are necessary for cellular localization and showed the stability of mutated SBDS protein and the mechanism of cellular localization.

The mechanism of nuclear localization is yet unknown. Ganapathi et al. [13] reported that NPM, which is a nucleolar protein involved in ribosomal RNA synthesis, was purified by co-immunoprecipitation with SBDS. However, SBDS was accumulated in the nucleoli independent of NPM [13]. Our data clearly show that the SBDS N-terminal Domain I is responsible for its nuclear localization. However, EGFP–SBDS Domain II was also found in the nuclei. Though the structure of SBDS Domain II is close to the C-terminal domain of *E. coli* RuvA, the function of its domain is still unknown [6, 31]. SBDS Domain I forms a compact structure, while on the other hand, SBDS Domain II forms an elongated structure [32]. Moreover, human SBDS Domain II structure is predicated as flexible by NMR data [32]. Structure flexibility may be essential for the conformation adjustments of modular protein; however, SBDS Domain II without any other domain might not form an original conformation. This is supported by the fact that both EGFP–SBDS Domain II–III and EGFP–SBDS Domain III were found in the cytosol. If the SBDS Domain II is responsible for nuclear localization, EGFP–SBDS Domain II–III should localize to the nucleus. We believe that nuclear localization of EGFP–SBDS Domain II was artificial.

We also analyzed 8 mutations within SBDS Domain I. The amount of mutated SBDS proteins, except SBDS D70N, was smaller than wild-type SBDS. Because these SBDS mutants were expressed by the CMV promoter, these results indicated that the mutated SBDS protein stability was reduced. Furthermore within SBDS Domain I, our data indicate that the mutation cysteine 31, which maps to the hydrophobic core of the FYSH domain as well as asparagine 34, is necessary for nuclear localization. The SBDS C31W mutation is predicted to result in a loss or reduction of protein instability confirmed by its low expression in HeLa cells. The amount of the SBDS N34I mutant is also decreased, although protein stability is also a potential explanation for the inability to detect nuclear localization. This is unlikely to be the case, as treating the cells with the proteasome inhibitor, Lactacystin, which results in an increase in protein amount, did not affect cytosolic expression of these mutant proteins. These results indicate that the FYSH domain, and specifically with cysteine 31 and asparagine 34, is necessary for SBDS nuclear localization.

SBDS protein was usually found in the nucleolus; however, both EGFP–SBDS and EGFP–SBDS Domain I were localized to the nucleus. It has been suggested that nucleoli targeting proteins may be a 2-step process: in the first step, nuclear localization signal mediates the initial translocation of the protein into the nuclei; in the second step, the protein translocates into the nucleolus [33]. It was reported that the nuclear localization signals and nucleolar localization signals of NO38 are distinct motifs [33]. Because the size of EGFP protein is 27 kDa, it may inhibit the second nucleolar localization and not nuclear localization. Also, we could not identify any obvious common sequence motifs between the N-terminal SBDS proteins and the nucleolar localization signals previously identified in RPS19 and NO38 proteins (data not shown).

Recently, Kayed et al. [34] reported that SBDS protein is localized to the cytosol in pancreatic cancer and chronic pancreatitis. Orelio et al. [11] reported that SBDS protein is

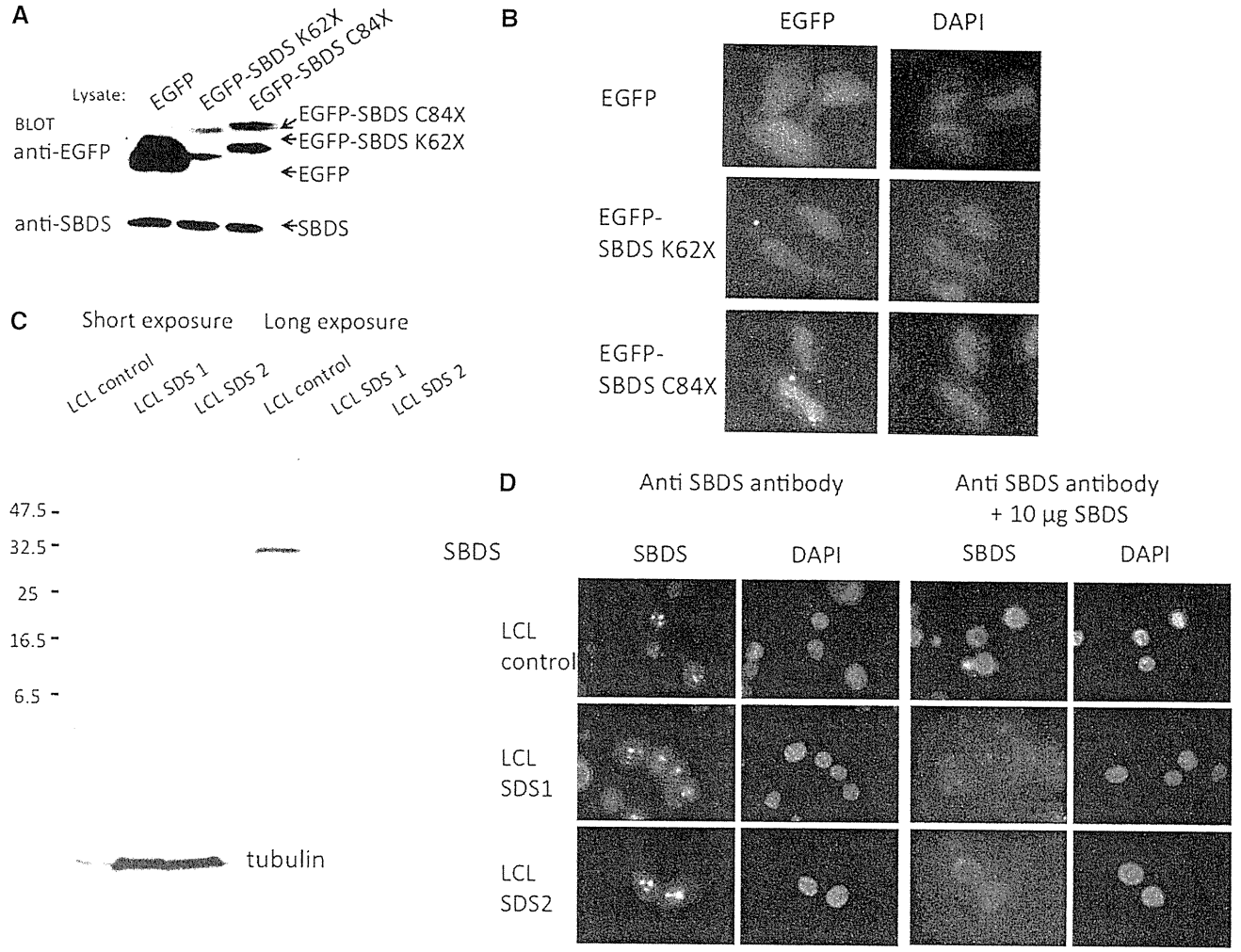


Fig. 4 N-terminal immature SBDS peptides are localized to nucleoli in SDS patients. **a** Expression of EGFP–SBDS K62X and EGFP–SBDS C84X. Cell lysates were detected with anti-SBDS antibody and anti-pTAG antibody. **b** N-terminal 61 amino acid residue of SBDS is sufficient to localize to the nuclei. EGFP–SBDS K62X and EGFP–SBDS C84X were transfected into HeLa cells. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS and counterstained with DAPI. **c** SBDS protein detection in SDS LCL cells. Control (LCL-control) and SDS patients-derived LCL cell (LCL-SDS1, 2) lysates were separated with SDS-PAGE, and SBDS protein was detected with Western blot. **d** Localization of N-terminal immature peptide in SDS patients. Control (LCL-control) and SDS-derived LCL cells (LCL-SDS1, 2) were fixed with 4% paraformaldehyde in PBS and incubated with affinity purified anti-SBDS antibody in the presence or absence of 10 μg of recombinant SBDS protein. Then, FITC conjugated anti-rabbit antibody was treated

found in the cytoplasm in the differentiated neutrophil. It is yet unknown how SBDS protein localizes to the nuclei. When EGFP–SBDS protein and EGFP–SBDS Domain I were overexpressed, the localization of endogenous SBDS protein was changed from the nucleic fraction to the cytosolic fraction. These results indicate that EGFP–SBDS and EGFP–SBDS Domain I compete with endogenous SBDS protein in 32Dcl3 and HeLa cells. Moreover, these results suggest that some shuttling protein may involve SBDS nuclear localization.

A majority of SDS patients have two common mutations, c.183C>T, and c.258+2T>C. These mutations result in immature N-terminal truncated peptide, p.K62X and p.C84fsX3. It is plausible that the N-terminal immature peptide localized to the nucleus in SDS patients, because EGFP–SBDS K62X and EGFP–SBDS C84X proteins were detected in nuclei as Orelio et al. [11] reported. It is very interesting that anti-SBDS antibody-specific signals were accumulated in the nucleoli of both control and SDS patients-derived LCL cells. These signals disappeared in the presence of recombinant SBDS protein. Austin et al. [12] reported that no signal was detected in the SDS patient-derived cell line by immunofluorescence. Austin's antibody was generated with C-terminal peptide; however, our antibody was made with recombinant SBDS protein. We could not isolate the 6- or 8-kDa peptide from patients' sample by immunoprecipitation (data not shown), because the reactivity of our anti-SBDS antibody to SBDS Domain

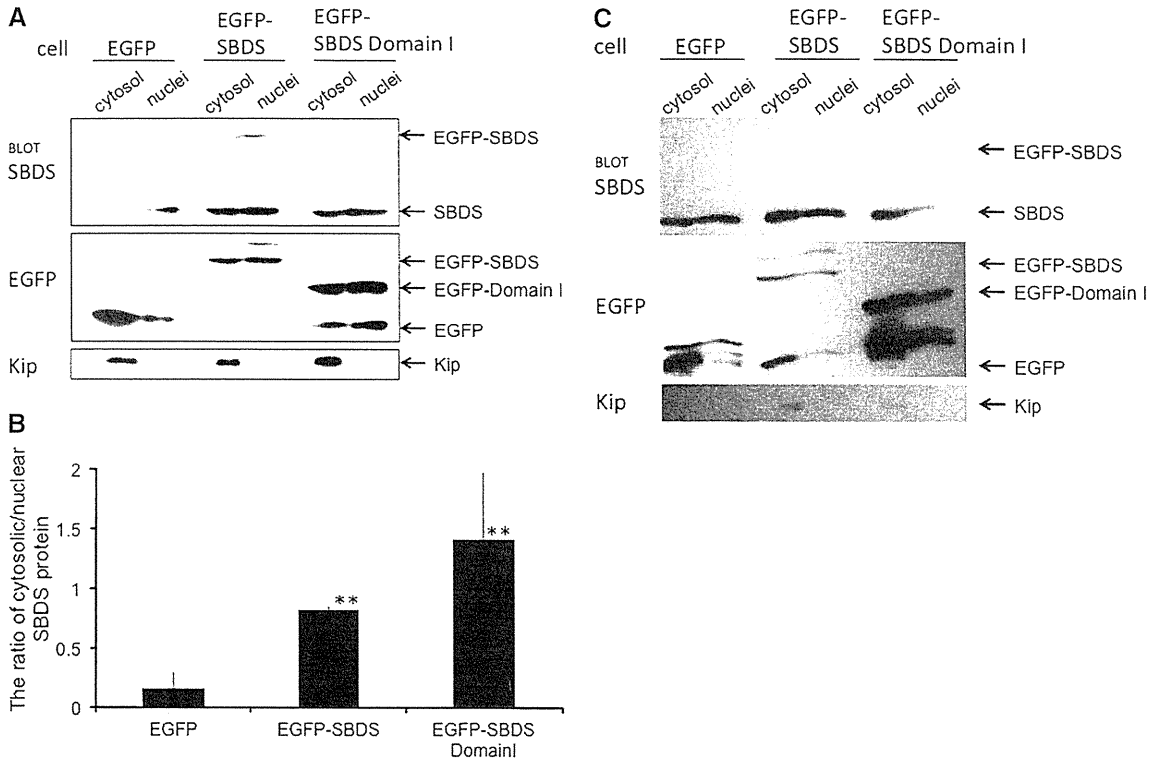


Fig. 5 N-terminal SBDS Domain I competes with endogenous SBDS nuclear localization. **a** The distribution of endogenous SBDS protein was analyzed in 32Dcl3/EGFP, 32Dcl3/EGFP–SBDS and 32Dcl3/EGFP–SBDS Domain I cell lines. The nuclear and cytosolic fractions were separated, and these extracts were subjected to SDS-PAGE and Western blot. **b** Schematic representation of the ration of cytosolic/nucleic SBDS distribution intensities from **a**. The ratio of cytosolic/nucleic SBDS protein was quantified for 3 independent experiments. $^{**}P < 0.05$. **c** The distribution of endogenous SBDS protein was analyzed in transiently expressed EGFP, EGFP–SBDS and EGFP–SBDS Domain I in HeLa cells. The nuclear and cytosolic fractions were separated, and these extracts were subjected to SDS-PAGE and Western blot

I was very poor in the Western blot (Fig. 5a). Because our antibody could not detect any signals in the SBDS shRNAi knockdown Jurkat cells (Supplementary data), our anti-SBDS antibody would react with only SBDS protein and SBDS-derived peptide specifically. These results indicated that the fluorescent signal that was detected in LCL-SDS patient cells referred to SBDS N-terminal peptides. These results suggest that the truncated immature polypeptide, which are expressed by mutation of c.183C>T, and c.258+2T>C, would accumulate in the nucleoli. This is the first report of the expression profile of immature SBDS protein in SDS and we also showed that Domain I competes with endogenous SBDS nuclear localization. It is plausible that SBDS N-terminal peptides interfere with SBDS protein localization to the nuclei. In addition, this competition of nuclear localization of SBDS might involve in SDS pathogenesis.

In summary, we have demonstrated that SBDS N-terminal Domain I is important for nuclear localization, and two amino acid residues, 31C and 34N, in N-terminal SBDS protein are necessary for nuclear localization. Moreover, the shuttling protein may be involved in nuclear localization by binding to N-terminal Domain I region. The amounts of mutated SBDS proteins were dramatically reduced, and this lower expression would affect cellular function of SBDS. Further study of the shuttling protein is warranted.

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INVITED REVIEW

Natural history of Upshaw–Schulman syndrome based on *ADAMTS13* gene analysis in Japan

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Summary. Upshaw–Schulman syndrome (USS) is an extremely rare hereditary deficiency of ADAMTS13 activity, termed congenital TTP. The clinical signs are usually mild during childhood, often with isolated thrombocytopenia. But their symptoms become more evident when patients have infections or get pregnant. We identified 43 USS-patients in Japan, who ranged in age from early childhood to 79 years of age. Analysing the natural history of these USS patients based on *ADAMTS13* gene mutations may help characterise their clinical phenotypes. Severe neonatal jaundice that requires exchange blood transfusion, a hallmark of USS, was found in 18 of 43 patients (42%). During childhood, 25 of 43 patients were correctly diagnosed with USS without gender disparity. These 25 patients were categorised as having 'the early-onset phenotype'. Between 15 and 45 years of age, 15 were correctly diagnosed, and, interestingly, they were all female. The remaining three patients were male and were diagnosed when they were older than 45 years of age, suggesting that they were 'the late-onset phenotype'. Two of these three males developed sudden overt TTP when they were 55 and 63 years old, respectively. These two men had two different homozygous *ADAMTS13* gene mutations, p.R193W/p.R193W and p.C1024R/p.C1024R, respectively. Both of which were not discovered in the US or Western countries. *In vitro* expression studies showed that these two proteins were consistently secreted into the culture medium but to a lesser extent and with reduced activity compared to the wild-type protein. Our results indicate that 'the late-onset phenotype' of USS is formed with ethnic specificity.

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Introduction

Upshaw–Schulman syndrome (USS) is an hereditary deficiency in the activity of von Willebrand factor-cleaving protease (VWF-CP) [1], termed ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs 13) [2–4]. In the absence of ADAMTS13, unusually large VWF multimers (UL-VWFMs) released from vascular endothelial cells are not cleaved appropriately, which induces platelet hyperagglutination under high shear stress [5]. Thus, USS is alternatively called congenital thrombotic thrombocytopenic purpura (TTP). On the other hand, approximately half of congenital atypical haemolytic uremic syndrome (aHUS) cases are caused by genetic mutations in complement regulatory factors, such as factor H, I, and B, and membrane cofactor protein, or thrombomodulin [6]. However, the majority of both TTP and HUS occur in the acquired form, and it has been said that TTP and HUS patients predominantly exhibit neurotropic and nephrotropic signs, respectively. Further, it is often difficult to discriminate between these two diseases in clinical practice [7–9] because both diseases are identified based on common pathological features termed thrombotic microangiopathies (TMAs), which are characterised by organ dysfunction due to platelet thrombi in the microvasculature, consumptive thrombocytopenia, and microangiopathic haemolytic anaemia (MAHA) [10].

The classic hallmarks of USS are severe neonatal jaundice with a negative Coombs test that requires an exchange blood transfusion and repeated childhood episodes of thrombocytopenia and MAHA that are reversed by infusions of fresh frozen plasma (FFP) [11]. However, recent studies indicated that the clinical signs of USS during childhood may be much milder than expected, and often only an isolated thrombocytopenia occurs, causing physicians to sometimes overlook this important disease [12].

Despite a lengthy history of clinical diagnoses of USS, only 10 years have passed since the disease-related enzyme, ADAMTS13, was discovered [3,4,13,14]. Furthermore, USS is an extremely rare disease, and to date, it is estimated that there have been approximately 100 patients worldwide [15]. In this regard, Nara Medical University has functioned as a TMA referral centre in Japan since 1998 and collected a large dataset of 919 patients with TMA between 1998 and 2008 [16]. In this registry, we identified 41 USS patients in 36 different families who ranged in age from early childhood to 79 years old. Subsequently, until the end of March 2011, we have identified two new USS-patients belonged to different families in Japan. Analysing the natural history of these 43 USS-patients will further our understanding of the clinical significance of ADAMTS13, which functions to regulate the size of platelet-thrombi that form in the microvasculature under high shear stress.

Historical backgrounds

In 1953, Dacie *et al.* [17] reviewed 12 patients with atypical congenital haemolytic anaemia and identified a 6-year-old girl who had experienced repeated episodes of severe jaundice, thrombocytopenia, haemolytic anaemia, and schistocytes since she was a newborn. Before first visiting Dacie, this patient had received a splenectomy but did not improve. She died of renal failure at 7 years of age. This patient was the third of four children, and both the first and second children were jaundiced at birth and died of haemorrhage at 2 years and 4 days old, respectively. The fourth child and the parents were asymptomatic. Thus, the authors concluded that these three patients must have a hitherto unrecognised hereditable blood disease.

In the absence of any known concept of TTP, in 1960, Schulman *et al.* [18] reported an 8-year-old girl who had no coagulation abnormalities but repeated bleeding episodes due to chronic thrombocytopenia and MAHA. These symptoms dramatically improved with fresh plasma infusions, suggested that the patient had a congenital deficiency in a 'platelet-stimulating factor' in her plasma. In 1978, Upshaw [19] reported the case of a 29-year-old female who had repeated episodes of thrombocytopenia and MAHA starting in childhood and was successfully treated with plasma infusions. Of note, both Schulman and Upshaw determined that plasma infusions successfully treated their patients. Rennard and Abe [20] reported a case that was originally identified by Upshaw with 'a slightly decreased level of plasma cold-insoluble globulin (fibronectin) during the acute phase', and proposed a nomenclature of USS for these types of patients. However, no correlation between the fibronectin levels and disease activity in USS patients was reported by Koizumi *et al.* [21] and Goodnough *et al.* [22], including Schulman's original case. Furthermore, after the thrombopoietin assay was established, Miura *et al.* [23] reported five Japanese USS patients with a normal plasma level of thrombopoietin. Thus, all the pathogenic features that were initially postulated for USS have been entirely excluded by subsequent investigations.

For this reason, the term USS was almost forgotten in 1997, when the assay for VWF-CP (now ADAMTS13) activity was established. Instead, the practical diagnostic term of 'chronic relapsing TTP (CR-TTP)' has been historically used. This term was coined by Moake *et al.* [24], who found that UL-VWFMs were present in the plasma of four CR-TTP patients including the Upshaw's case during the remission phase, but disappeared during the acute phase. In 1997, Furlan *et al.* [25] showed that four CR-TTP patients, who were distinct from the cases of Moake *et al.* [24], lacked VWF-CP activity, but did not examine the presence of ADAMTS13 inhibitors. However, it was retrospectively determined that two CR-TTP patients in both the case reports by Moake *et al.* [24] and Furlan *et al.* [25] had congenital TTP, while the remaining two cases in each report had acquired TTP. Under these circumstances, we revisited the term USS [11], which included analysing three Japanese patients with USS, and found that they uniformly had a severe deficiency in VWF-CP activity (determined based on the VWFM assay in the presence of 1.5 mol L^{-1} urea) and no evidence of inhibitors. The parents of these patients were asymptomatic and had moderately decreased VWF-CP activity (17–60% of normal), except for one carrier who had very low VWF-CP activity (5.6% of normal). Later, this carrier was shown to have a unique single nucleotide polymorphism (SNP), a p.P475S mutation in the *ADAMTS13* gene in one allele, which is very common among Japanese people (9.6% of normal individuals are heterozygous for the p.P475S mutation) [26].

In 2001, Levy *et al.* [3] provided solid evidence that linked congenital TTP or USS and *ADAMTS13* gene mutations, and simultaneously other research groups successfully purified this enzyme and/or cloned the encoding cDNA [2,4,13,14].

Patients, materials and methods

USS patients

Forty-three USS patients (28 females and 15 males) belonging to 38 different families and their family members were enrolled in this study.

Assays for plasma ADAMTS13 activity and ADAMTS13 inhibitors

Between 1998 and 2004, our laboratory examined ADAMTS13 activity using a classic VWFM assay in the presence of 1.5 mol L^{-1} urea following the method of Furlan *et al.* [27]. The detection limit of this assay was 3% of the normal control [11]. In 2005, Kato *et al.* [28] developed a novel chromogenic ADAMTS13-act-ELISA using a recombinant VWF substrate (termed GST-VWF73-His). The detection limit of this assay was 0.5% of the normal control [28]. Both assays had a high correlation, and since then, the VWFM assay was completely replaced with the act-ELISA. In our patients with USS, the ADAMTS13 activity was determined at least two different occasions, using their plasmas obtained at more than 2 weeks

after the last plasma infusion therapy. Further, in some experiments with normal individuals as described below, FRETS-VWF73 assays [29] were used.

The ADAMTS13 inhibitor titers were evaluated using the Bethesda method, and the values < 0.5 Bethesda U (BU) mL^{-1} were negative, but those between 0.5 and 1.0 BU mL^{-1} were assumed to be marginal.

Assay for IgG-type plasma ADAMTS13 binding antibody titers

Measurement of plasma anti-ADAMTS13 IgG antibody titers in USS-patients was performed as described by Ferrari *et al.* [30] with a slight modification. Briefly, the recombinant (r) ADAMTS13 was directly coated to micro-titer plates, and after blocking with Protein-Free Blocking Buffers (Pierce, Rockford, IL, USA), the coated plates were incubated with normal and patient plasma dilutions. The IgG-type antibody bound to rADAMTS13 was detected by using horseradish peroxidase-conjugated goat anti-human IgG (AbD Serotec, Kidlington, UK) with a TMB substrate kit (Thermo Scientific, Rockford, IL, USA) at absorbance 450 nm at room temperature for 15 min. The results were calculated as a ratio of sample OD at each dilution divided by normal plasma OD at each dilution. The IgG antibody titer of a sample corresponds to the last dilution at which the ratio is above the cut-off level. This assay kit was kindly provided from Drs Barbara Plaimauer and Friedrich Scheiflinger of Baxter BioScience. In our laboratory, 25 normal plasmas (15 males and 10 males, aged between 20 and 40 years) consistently showed the titer of IgG-type binding antibody with a < 25-fold dilution (shown as < 25 × in Table 1).

ADAMTS13 gene analysis

All DNA analyses of the *ADAMTS13* gene were performed as previously described [26], with permission from the Ethics Committees of both the sample-collecting hospitals and the institute that performed the gene analysis. Hereafter, the disease-causing mutations (DCMs) of ADAMTS13 are highlighted in bold.

Results

ADAMTS13 SNPs among Japanese individuals

The human *ADAMTS13* gene is located on chromosome 9q34. The gene consists of 29 exons, and the translated enzyme contains 1427 amino acid residues with a multi-domain structure [2,4]. To date, more than 10 SNPs in *ADAMTS13* have been identified worldwide [3,26]. Among these, Japanese people (n = 3616) had six SNPs with the following allele frequencies, respectively: p.T339R (exon 9) (2.7%), p.Q448E (exon 12) (19.2%), p.P475S (exon 12) (5.0%), p.P618A (exon 16) (2.7%), p.S903L (exon 21) (4.8%), and p.G1181R (exon 25). (2.2%) [31]. Both p.T339R and p.P618A are almost completely linked in the general Japanese population, but this linkage may not exist in the Caucasian population as some reports have described individuals carrying p.P618A but not p.T339R [32,33]. Plasma and rADAMTS13 with the Asian-specific SNP, p.P475S [26,34,35], has markedly reduced activity compared to the wild-type protein in both the VWFM assay in the presence of urea (1.5 mol L^{-1}) [26] and the FRETS-VWF73 assay in the absence of urea [36], although the contribution of this polymorphism to thrombotic diseases has not been determined.

Recently, we have analysed the nucleotide sequences of *ADAMTS13* in 128 individuals without a history of TTP and identified 14 rare nonsynonymous mutations. Interestingly, among these 14 mutations, three mutations of **p.I673F** (exon 17), **p.Q723K** (exon 18), and **c.3220del TACC**, were also found as DCMs for USS in this study (below). Thus, the remaining 11 mutations may or may not be associated with a reduced activity of plasma ADAMTS13 [31].

Natural history of 43 USS-patients in Japan

Until the end of March 2011, we identified 43 USS patients in 38 different families (Family USS-A∼LL) who ranged in age from early childhood to 79 years old. Hence, we present an up-dated natural history of these 43 USS patients together with their family members (Tables 1 and 2).

Family USS-A

Patient

One male (USS-A4) born in 1999.

Brief clinical data

The history of USS-A4 (*ADAMTS13* genotype: **p.R268P**/p.Q448E-**p.C508Y**) before he reached 5 years of age was previously described [11,26,37]. He currently receives biweekly FFP infusions (10 mL kg BW^{-1}) that are prepared from several fixed donors to prevent allergic reactions. He contracted the seasonal influenza A virus in 2010 and became severely ill with a reduced platelet count but did not develop overt TTP. He is currently in good clinical condition and has not had signs of renal or hepatic dysfunction. Both of his parents are asymptomatic carriers, but his father, aged 45 years old, has a **p.R268P**/p.P475S genotype and very low plasma ADAMTS13 activity at 5.6% of normal by the VWFM assay in the presence of 1.5 mol L^{-1} urea [26] and 3.6% by the chromogenic ADAMTS13-act-ELISA in the absence of urea (unpublished data).

Family USS-B

Patient

One female (USS-B3) born in 1986.

Table 1 Fourty-three Japanese patients with USS registered in Nara Medical University and their ADAMTS13 gene mutations

No.	Patient	Year of birth	Gender	ADAMTS13 Activity (%)	ADAMTS13 Inhibitor (BU mL^{-1})	ADAMTS13 Titer of IgG-type binding antibody (Year of examination)	*ADAMTS13* gene mutations Father's origin DCM	Father's origin Missense SNP	Mother's origin DCM	Mother's origin Missense SNP	Reference numbers and remarks
1	A4	1999	M	< 0.5	< 0.5	< 25 × (2001), < 25 × (2011)	p.R268P		p.C508Y	p.Q448E	11,26,37
2	B3	1986	F	< 0.5	< 0.5	< 25 × (2005), < 25 × (2008)	p.Q449X		p.Q449X		11,26,38
3	C3	1972	M	< 0.5	< 0.5	< 25 × (1999)	c.414+1G>A		c.414+1G>A		39,40
4	D4	1978	F	< 0.5	< 0.5	25 × (2001), < 25 × (2009)	p.I673F		c.414+1G>A		40,41
5	E4	1985	M	< 0.5	< 0.5	< 25 × (2001), < 25 × (2011)	p.I673F		p.C908Y		40
6	F3	1993	M	0.6	< 0.5	< 25 × (2002), < 25 × (2009)	p.R193W		c.1244+2T>G		40
7	G3	1987	F	< 0.5	< 0.5	< 25 × (2009)	p.R1123C		c.686+1G>A		40
8	H3	1951	M	0.6	< 0.5	*	p.A250V		c.330+1G>A		42
9	I4	1972	M	< 0.5	< 0.5	< 25 × (2003), < 25 × (2009)	p.H234Q		p.R1206X		43
10	J3	1977	F	< 0.5–0.8	< 0.5	< 25 × (2000)	p.R312C		c.3198delCT		†
11	J4	1979	M	< 0.5	< 0.5	< 25 × (2000)	p.R312C		c.3198delCT		†
12	K3	1976	F	< 0.5–0.7	< 0.5	200 × (2003), 400 × (2011)	p.Y304C		p.G525D	p.T339R, p.Q448E, p.P618A	12
13	K4	1978	F	< 0.5	< 0.5	25 × (2003), 100 × (2011)	p.Y304C		p.G525D	p.T339R, p.Q448E, p.P618A	12
14	L2	1967	F	< 0.5	< 0.5	< 25 × (2003)	p.Q1302X	p.P618A	p.R125VfsX6	p.T339R, p.Q448E	12
15	L3	1972	F	< 0.5	< 0.5	< 25 × (2003)	p.Q1302X	p.P618A	p.R125VfsX6	p.T339R, p.Q448E	12
16	M3	1969	F	< 0.5	< 0.5	< 25 × (2002)	p.R193W		p.R349C		12
17	M4	1971	F	< 0.5	< 0.5	< 25 × (2002)	p.R193W		p.R349C		12
18	N6	1986	F	< 0.5	< 0.5	< 25 × (1999), < 25 × (2005)	p.H234R	p.P475S	c.3220delTACC		11,37
19	O4	1958	F	< 0.5	< 0.5	< 25 × (2009)	p.I178T		p.Q929X		12
20	P3	1971	M	< 0.5	< 0.5	< 25 × (2003), < 25 × (2005)	p.C908Y				45 *de novo* mutation (p.C322G, p.T323R, p.F324L)
21	Q1	1983	M	< 0.5–0.7	< 0.5	25 × (2004), 25 × (2009)	p.G227R	p.G1181R	p.C908Y		46
22	Q2	1988	M	< 0.5	< 0.5	< 25 × (2007), < 25 × (2009)	p.G227R	p.G1181R	p.C908Y		46
23	R5	1982	F	< 0.5	< 0.5	25 × (2005)	p.R193W		p.A606P	p.T339R, p.Q448E, p.P618A	12
24	S3	1982	M	0.9	< 0.5	< 25 × (1998)	*		*		†
25	T4	1981	F	< 0.5	< 0.5	< 25 × (2009)	c.3220delTACC		c.3220delTACC		47
26	U3	1990	F	< 0.5	< 0.5	< 25 × (2009)	c.2259delA		c.2259delA		†
27	V3	1983	F	< 0.5	< 0.5	< 25 × (2009)	p.W1081X		p.R193W		†
28	W4	1990	F	< 0.5	< 0.5	< 25 × (2005), < 25 × (2009)	p.G550R	p.Q448E	*	p.P475S	†
29	X5	1963	F	< 0.5	< 0.5	200 × (2004)	*	p.G1181R	*	p.P475S	†
30	Y3	1960	F	< 0.5	< 0.5	< 25 × (2005)	p.G385E		p.R1206X		†
31	Z3	1971	F	< 0.5	< 0.5	< 25 × (2006), < 25 × (2009)	p.R193W		p.R193W		12
32	AA3	1987	F	< 0.5	< 0.5	200 × (2006)	*		*		†
33	BB3	1947	M	< 0.5	< 0.5	< 25 × (2006)	p.R193W		p.R193W		†
34	CC5	2004	M	< 0.5	< 0.5	< 25 × (2007)	p.Q723K		p.R398C		†
35	DD5	2007	F	< 0.5	< 0.5	25 × (2007), < 25 × (2009)	p.R268P		p.Y304C		†
36	EE4	2003	M	< 0.5	< 0.5	200 × (2008), 200 × (2011)	c.2259delA		c.2259delA		†
37	FF3	1991	F	< 0.5	< 0.5	< 25 × (2008)	p.Q449X		p.Q449X		48

Table 1 (Continued).

No.	Patient	Year of birth	Gender	ADAMTS13 Activity (%)	ADAMTS13 Inhibitor (BU mL^{-1})	ADAMTS13 Titer of IgG-type binding antibody (Year of examination)	*ADAMTS13* gene mutations Father's origin DCM	Father's origin Missence SNP	Mother's origin DCM	Mother's origin Missence SNP	Reference numbers and remarks
38	GG2	1931	M	2.4–3.4	< 0.5	< 25 × (2008)	p.C1024R		p.C1024R		†
39	HH4	2003	F	< 0.5	< 0.5	< 25 × (2008)	p.Q449X		c.4119delG		†
40	II3	1977	F	< 0.5	< 0.5	50 × (1998), 50 × (2009)	*		*		†
41	JJ3	1980	M	< 0.5	< 0.5	< 25 × (2009)	c.1885delT		p.C908Y		Parent's origin is unknown†
42	KK3	1976	F	< 0.5	< 0.5	< 25 × (2011)	*		*		†
43	LL4	1981	F	< 0.5–1.8	< 0.5–1.4	> 400 × (2002), 200 × (2011)	p.C438S		p.G909R	p.T339R, p.Q448E, p.P618A	†

DCM, disease causing mutation; SNP, single nucleotide polymorphism; USS, Upshaw–Schulman syndrome. *Undetermined. †Unpublished.

Brief clinical data

USS-B3 (*ADAMTS13* genotype: **p.Q449X/p.Q449X**) is an only child who was born in Hokkaido to non-consanguineous parents. Her history prior to reaching 5 years of age was previously described [11,26,38]. Since childhood, she has received prophylactic FFP infusions. As a consequence, she was infected with hepatitis C and has received interferon therapy on two different occasions. In both instances, accelerated thrombocytopenia was observed despite the regular prophylactic FFP infusions. Furthermore, during her early childhood, she received DDAVP (1-desamino-8-D-arginine vasopressin) infusion once that immediately aggravated her clinical signs, including haematuria and thrombocytopenia. Currently, her renal function is normal and her liver function is well preserved (communication with Dr Mutsuko Konno). Her parents initially stated that they had a non-consanguineous marriage. However, a subsequent ancestral analysis revealed that two great-grandparents of USS-B3 on the paternal and maternal sides migrated from the same area (a small fisherman's village) of Iwate to Hokkaido at the end of the 19th century when Hokkaido was an undeveloped island, and the pioneers settled from the Japanese mainland (Honshu). This fisherman's village is located in the northern part of Honshu (Tohoku) facing the Pacific ocean, an area severely damaged several times by earthquake and tsunami – most recently in March 11, 2011. In the old days, this small village was isolated from neighbours, and was surrounded by mountains, suggesting that there were many consanguineous marriages within this village.

Family USS-C

Patient

One male (USS-C3) born in 1972.

Brief clinical data

USS-C3 (*ADAMTS13* genotype: **c.414+1G>A/c.414+1G>A**) is the last of four children to consanguineous parents (first cousins). Notably, the patient's elder brother (third sibling) died of melena soon after birth. The history of this patient was previously described [39,40]. At 8 years of age, USS-C3 was clinically diagnosed with USS. Since then, he has received prophylactic FFP (160 mL) infusions every 2–4 weeks. However, his renal function due to chronic nephritis gradually deteriorated, and in 1995 he required continuous ambulatory peritoneal dialysis (CAPD). Because of repeated peritonitis associated with CAPD, his therapy for renal insufficiency was changed to haemodialysis (three times per week) in 1999. However, his cardiac function decreased, and he eventually died of chronic heart failure in 2010 at 38 years of age.

Family USS-D

Patient

One female (USS-D4) born in 1978.

Brief clinical data

USS-D4 (*ADAMTS13* genotype: **p.I673F/c.414+1G>A**) was born as the second of 2 children to non-consanguineous parents. Her history was previously described [40,41].

Family USS-E

Patient

One male (USS-E4) born in 1985.

Brief clinical data

USS-E4 (*ADAMTS13* genotype: **p.I673F/p.C908Y**) was born as the second of three children to non-consanguineous parents. His history was previously described [40]. The third sibling had Down's syndrome and died of an unknown cause soon after birth.

Family USS-F

Patient

One male (USS-F3) born in 1993.

Brief clinical data

USS-F3 (*ADAMTS13* genotype: **p.R193W/c.1244+2T>G**) was born as the first of three children to non-consanguineous parents. His history was previously described [40]. He currently receives 120 mL FFP infusions when he develops an occasional haemolytic crisis.

Family USS-G

Patient

One female (USS-G3) born in 1987.

Table 2 Clinical course of 43 Japanese patients with USS

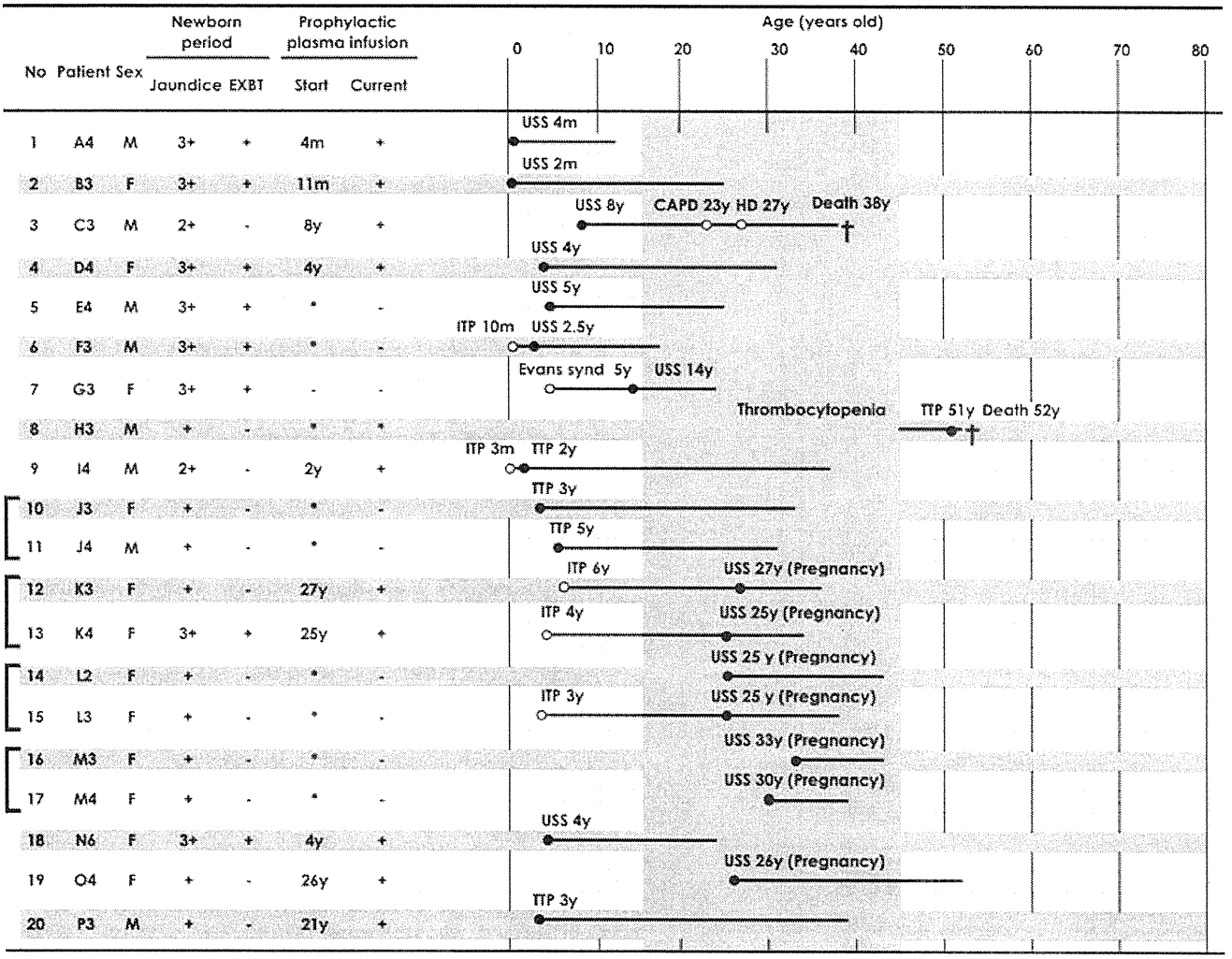
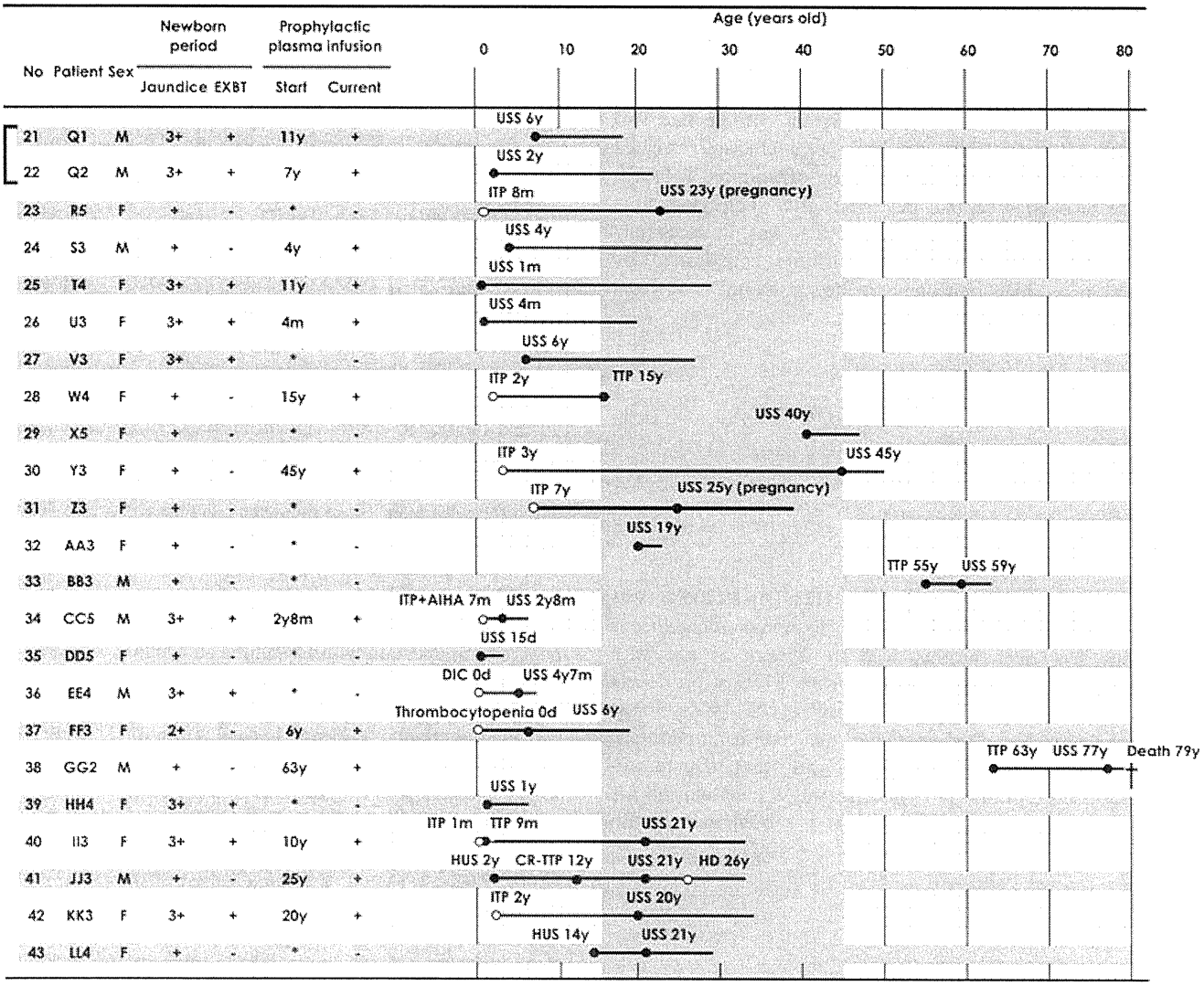


Table 2 Continued



EXBT, exchange blood transfusion; TTP, thrombotic thrombocytopenic purpura; HUS, hemolytic uremic syndrome; ITP, idiopatic thrombocytopenic purpura; DIC, disseminated intravasclar coagulation; AIHA, autoimmune hemolytic anemia; CAPD, continuous ambulatory peritoneal dialysis; HD, hemodialysis; USS, Upshaw–Schulman syndrome. *Undertermined.

Brief clinical data

USS-G3 (*ADAMTS13* genotype: **p.R1123C/c.686+1G>A**) was born as the third of four children to non-consanguineous parents. Her history before she reached 14 years of age was previously described [40]. Of note, her elder sister (second child) had severe jaundice soon after birth and received an exchange blood transfusion. She died of intracranial bleeding after a trivial traffic accident when she was 8 years old. USS-G3 received prophylactic FFP infusions after she was diagnosed with USS, but the infusion intervals gradually increased. Under these circumstances, she became pregnant at 21 and 22 years of age. During the first pregnancy, she had a spontaneous abortion at 5 weeks of gestation. During the second pregnancy, she delivered a premature baby girl (1446 g BW) at 35 weeks of gestation after a caesarean section under extensive prophylactic FFP infusions (normal Japanese female baby at 35 weeks of gestation has a BW of median 2173 g). Interestingly, many of the placental small vessels were occupied with hyaline thrombosis (communication with Dr Michiko Kajiwara, details will be published elsewhere by the physicians in charge).

Family USS-H

Patient

One male (USS-H3) born in 1951.

Brief clinical data

USS-H3 (*ADAMTS13* genotype: **p.A250V/c.330+1G>A**) was born to non-consanguineous parents, but the details were unclear [42]. At 51 years of age, he visited a nearby

hospital because of a convulsive seizure after haemorrhoidectomy where he was diagnosed with TTP. He had an episode of childhood thrombocytopenia, but there is no additional information. After 51 years of age, he had two episodes of overt TTP, and both were efficiently treated with FFP infusions. In July 2002, he experienced a fourth episode of overt TTP that developed after cholecystectomy, followed by gastrointestinal bleeding that was unsuccessfully treated with FFP infusions and further complicated by renal failure, which ultimately resulted in death at 52 years of age.

Family USS-I

Patient

One male (USS-I4) born in 1972.

Brief clinical data

USS-I4 (*ADAMTS13* genotype: **p.H234Q/p.R1206X**) was born as the second of two children to non-consanguineous parents. His elder brother died at 2 years of age with clinical signs that were compatible with TTP, as previously described [43]. At the age of 3 months, USS-I4 developed thrombocytopenia after receiving the diphtheria/pertussis/tetanus vaccine and was diagnosed with idiopathic thrombocytopenic purpura (ITP). Since he was 2 years old, he has experienced repeated overt TTP that has been treated with plasma infusions.

Family USS-J

Patients

One female (USS-J3) born in 1977 and one male (USS-J4) born in 1979.

Brief clinical data

USS-J3 (*ADAMTS13* genotype: **p.R312C/c.3198delCT**) and -J4 (*ADAMTS13* genotype: **p.R312C/c.3198delCT**) are the first and second of three children to non-consanguineous parents, respectively. For these two patients, severe jaundice was not noted during the newborn period. At 3 years of age, USS-J3 developed a cold followed by purpura with thrombocytopenia and was diagnosed with disseminated intravascular coagulation (DIC). Since then, she has experienced repeated episodes of thrombocytopenia and haemolytic anaemia, and was diagnosed with CR-TTP at 6 years of age. USS-J4 had an episode of purpura and thrombocytopenia when he was 5 years old. In 2000, both patients were shown to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. These two patients were not given prophylactic FFP infusions.

Family USS-K

Patients

Two females (USS-K3 born in 1976 and USS-K4 born in 1978).

Brief clinical data

Patients USS-K3 (*ADAMTS13* genotype: **p.Y304C**/p.T339R-p.Q448E-**p.G525D**-p.P618A) and -K4 (*ADAMTS13* genotype: **p.Y304C**/p.T339R- p.Q448E-**p.G525D**-p.P618A) were the first and second of two children of non-consanguineous parents, respectively. The history of these two patients was previously reported [12]. In 2003, USS-K3 became pregnant at 27 years old and developed overt TTP at 25 weeks of gestation. She experienced intrauterine foetal death followed by a caesarean delivery with a hysterectomy. On this occasion, she was diagnosed with USS. Since then, she has received prophylactic FFP infusions (80–120 mL) every 4 weeks in an out-patient clinic with a good clinical course. However, at the end of 2010, she had H1N1 influenza A virus infection that remarkably aggravated thrombocytopenia and was hospitalised for treatment (communication with Dr Junji Tomiyama).

In 2003, USS-K4, the younger sibling, became pregnant 2 months after her elder sister. She developed mild thrombocytopenia without significant clinical signs at 22 weeks of gestation. She underwent ADAMTS13 analysis, which confirmed a diagnosis of USS. While being treated with FFP infusions, she delivered a premature baby by a caesarean section [12]. Since then, she has received FFP infusions of 80 mL every 3 weeks. In 2008, 5 years after her first pregnancy, USS-K4 became pregnant for the second time and received more frequent FFP infusions (160 mL biweekly). At 29 weeks of gestation, her platelet count suddenly and severely dropped. Thus, at 30 weeks of gestation, a caesarean section was performed, and she delivered a baby (1522g BW) with congenital heart failure due to a ventricular septum defect (details will be published elsewhere by the physicians in charge).

Family USS-L

Patients

Two females (USS-L2 born in 1967 and USS-L3 born in 1972).

Brief clinical data

Both patients USS-L2 (*ADAMTS13* genotype: p.618A-**p.Q1302X/p.R125fsX6**-p.T339R-p.Q448E) and -L3 (*ADAMTS13* genotype: p.618A-**p.Q1302X/p.R125fsX6**-p.T339R-p.Q448E) were born as the second and fifth of five children to non-consanguineous parents. The history of these two patients was previously described [12]. At 27 years of age, USS-L2 became pregnant. At 27 weeks of gestation, she had intrauterine foetal death due to a suspected diagnosis of HELLP (haemolysis, elevated liver-enzymes, low platelets) syndrome. However, she

subsequently had four children who were all premature and uniformly born at approximately 30 weeks of gestation by a caesarean section with oral aspirin. Patient USS-L3, the younger sister of USS-L2, was diagnosed with ITP at 3 years of age. She had two pregnancies at 25 and 27 years of age. However, she lost both babies at 23 and 24 weeks of gestation, respectively, under a suspected diagnosis of 'habitual abortion'.

Family USS-M

Patients

Two females (USS-M3 born in 1969 and USS-M4 born in 1971).

Brief clinical data

Patients USS-M3 (*ADAMTS13* genotype: **p.R193W/p.R349C**) and USS-M4 (*ADAMTS13* genotype: **p.R193W/p.R349C**) were born as the second and third of four children to non-consanguineous parents. The history of USS-M3 was previously described [12]. USS-M3 was primigravida at 33 years of age, and at 20 weeks of gestation she miscarried with overt TTP. The history of her younger sister, USS-M4, was also previously reported [12]. However, recently Kato *et al.* [44] reported a more detailed account of the pregnancy of USS-M4, to which we have to make some corrections. According to that report, USS-M4 became primigravida at 28 years of age. Until 28 weeks of gestation, the pregnancy was uneventful when she suddenly stopped feeling foetal movement, resulting in intrauterine foetal death and a subsequent diagnosis of HELLP syndrome. One year later, at the age of 29, she became pregnant for the second time. She was diagnosed with ITP and treated with prednisolone therapy until 37 weeks of gestation, but with incremental low platelet counts (approximately 23×10^9 L^{-1}). Soon after this, she underwent a caesarean section after receiving concentrated platelet infusions that transiently increased her platelet counts to 96×10^9 L^{-1}. As a result, she delivered a healthy baby. At 32 years of age, she became pregnant for the third time. At 20 weeks of gestation, she developed DIC followed by multi-organ failure, despite extensive treatments, including platelet transfusions. By this time, she had been diagnosed with USS and had undergone ADAMTS13 analysis, along with her elder sister, USS-M3. At the age of 36, USS-M4 became pregnant for a fourth time. With extensive FFP infusions, she continued her pregnancy until 36 weeks of gestation and delivered a healthy baby (2506 g BW) by natural birth with a skin incision [44].

Family USS-N

Patient

One female (USS-N6) born in 1986.

Brief clinical data

Patient USS-N6 (*ADAMTS13* genotype: p.**H234R**-p.P475S/**c.3220delTACC**) was born as the last of four children to non-consanguineous parents. She had a history of severe neonatal jaundice and childhood thrombocytopenia. Her clinical data were previously reported [11,37]. Of note, she developed a thrombotic occlusion of the left carotid artery at 11 years of age that resulted in right hemiparesis. Subsequently, she developed hypertension and proteinuria, but these clinical signs have significantly improved during a long clinical course with prophylactic FFP infusions, although some neurological sequelae have persisted (communication with Dr Seiji Kinoshita).

Family USS-O

Patient

One female (USS-O4) born in 1958.

Brief clinical data

Patient USS-O4 (*ADAMTS13* genotype: **p.I178T/p.Q929X**) was the second of two children to non-consanguineous parents. The history of USS-O4 was previously described [12]. At the age of 26, USS-O4 became pregnant. At 23 weeks of gestation, she developed thrombocytopenia and delivered a premature infant at 25 weeks of gestation who died soon after birth. After delivery, she developed overt TTP that was rescued with plasma exchange. At 31 years of age, she became pregnant for the second time while receiving prophylactic FFP infusions every 1–2 weeks. At 8 weeks of gestation, she developed proteinuria and thrombocytopenia, and therefore received more frequent FFP infusions. At 36 weeks of gestation, she delivered a healthy baby girl.

Family USS-P

Patient

One male (USS-P3) born in 1971.

Brief clinical data

The clinical data for patient USS-P3 (*ADAMTS13* genotype: **p.C908Y/p.C322G-p.T323R-p.F324L**, *de novo* mutation) were previously described [45]. Briefly, USS-P3 was the second of four children to non-consanguineous parents. The first and fourth siblings died of an abortion at 6 and 22 weeks of gestation, respectively, due to unknown causes. At 3 years of age, USS-P3 had clinical signs of overt TTP, which was efficiently treated with FFP infusions. He was repeatedly treated with FFP infusions when overt TTP developed. Thus, after 21 years of age, the prophylactic FFP infusions were continued.

Family USS-Q

Patients

Two males, (USS-Q1) born in 1983 and (USS-Q2) born in 1988.

Brief clinical data

Patients USS-Q1 (*ADAMTS13* genotype: **p.G227R**-p.G1181R/**p.C908Y**) and -Q2 (*ADAMTS13* genotype: **p.G227R**-p.G1181R/**p.C908Y**) were the first and third of three children to non-consanguineous parents. Their detailed clinical data during childhood were reported in 1990 [46].

Family USS-R

Patient

One female (USS-R5) born in 1982.

Brief clinical data

USS-R5 (*ADAMTS13* genotype: **p.R193W**/p.T339R-p.Q448E-**p.A606P**-p.P618A) was the last of three children to non-consanguineous parents. The history of USS-R5 was previously reported [12]. Briefly, at 23 years of age, she became pregnant. At 23 weeks of gestation, she developed mild thrombocytopenia, and at 31 weeks of gestation, she had sudden intrauterine foetal death. After a caesarean section, she developed overt TTP, which was treated with plasma exchange and steroids. On this occasion, she was diagnosed with USS after her ADAMTS13 activity and ADAMTS13 inhibitor status were analysed. This patient did not receive prophylactic FFP infusions.

Family USS-S

Patient

One male (USS-S3) born in 1982.

Brief clinical data

USS-S3 (*ADAMTS13* genotype: undetermined) was born to non-consanguineous parents. Neither his childhood nor family history have been obtained. The patient was clinically diagnosed with USS at a nearby hospital when he was 4 years old. Since then, he has received prophylactic FFP infusions every 1 weeks at the same hospital. In 2002, USS-S3 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. Furthermore, the ADAMTS13 activities for his father and mother were 34.2% and 47.6%, respectively. This family has not been examined for *ADAMTS13* gene mutations.

Family USS-T

Patient

One female (USS-T4) born in 1981.

Brief clinical data

USS-T4 (*ADAMTS13* genotype: **c.3220delTACC/c.3220delTACC**) was born as the second of two children to non-consanguineous parents. Soon after birth, she developed severe neonatal jaundice and received exchange blood transfusion for three times [47]. One month after birth, she developed haematuria with thrombocytopenia, which led to a clinical diagnosis of USS. She received DDAVP infusion once at the age of 4, by which her platelet count promptly dropped and her clinical signs were aggravated, in accord with a transient disappearance of larger VWFMs from plasma [47]. Thus, she has received prophylactic FFP infusions every 2 weeks since 1992.In 1998, USS-T4 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. She had a homozygous *ADAMTS13* gene mutation of **c.3220del TACC/c.3220delTACC** (exon 24).

Family USS-U

Patient

One female (USS-U3) born in 1990.

Brief clinical data

USS-U3 (*ADAMTS13* genotype: **c.2259delA/c.2259delA**) was born as the second of two children to consanguineous parents (second cousins). Soon after birth, she developed severe neonatal jaundice that required an exchange transfusion. She was clinically diagnosed with USS at 4 months of age. In 1998, USS-U3 was confirmed to have a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. She was homozygous for an *ADAMTS13* gene mutation of **c.2259delA/c.2259delA** (exon 19). This patient has continued prophylactic FFP infusions.

Family USS-V

Patient

One female (USS-V3) born in 1983.

Brief clinical data

USS-V3 (*ADAMTS13* genotype: **p.W1081X/p.R193W**) was born as the second of two children to non-consanguineous parents. Soon after birth, she developed severe neonatal jaundice that required an exchange blood transfusion. She was clinically diagnosed with USS at 4 years of age. In 1998,

USS-V3 was confirmed to have a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. She had a compound heterozygous *ADAMTS13* gene mutation with **p.W1081X** (exon 24) from her father and **p.R193W** (exon 6) from her mother. The patient has been administered FFP infusions on demand.

Family USS-W

Patient

One female (USS-W4) born in 1990.

Brief clinical data

USS-W4 (*ADAMTS13* genotype: p.Q448E-**p.G550R**/p.P475S) was born as the second of two children to non-consanguineous parents. She did not have episodes of severe jaundice as a newborn. At 2 years of age, she developed pneumonia followed by thrombocytopenia. Since then, she has had repeated episodes of thrombocytopenia and haemolytic anaemia that have coincided with various infections, resulting in a diagnosis of Evans syndrome. In 2005, USS-W4 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. *ADAMTS13* gene analysis in USS-W4 suggested that she was a compound heterozygote with a **p.G550R** (exon 14) mutation from her father and an unidentified DCM from her mother. This patient has received prophylactic FFP infusions every 2 weeks.

Family USS-X

Patient

One female (USS-X5) born in 1963.

Brief clinical data

USS-X5 (*ADAMTS13* genotype: p.G1181R/p.P475S) was the last of four children to non-consanguineous parents. She did not have severe neonatal jaundice or childhood thrombocytopenia. She had two pregnancies at the ages of 24 and 26 years that yielded two children. During her first pregnancy, she had pregnancy-induced hypertension, but the details are unknown. At 32 years of age, she developed nephrotic syndrome, followed by repeated haemolytic anaemia and thrombocytopenia of an unknown cause. None of the laboratory markers were indicative of connective tissue disease. She underwent a splenectomy at the age of 36. In 2004, she had a relapse of nephrotic syndrome with haemolytic anaemia and thrombocytopenia that was treated with high-dose steroid therapy with limited success. At this time, her plasma ADAMTS13 activity levels and ADAMTS13 inhibitor status were examined, and she was determined to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. The same results were obtained 6 months later with a different plasma specimen. An *ADAMTS13* gene analysis in USS-X5 identified no DCMs, but revealed two SNPs of p.P475S from her mother and p.G1181R from her father. In 2007, she developed systemic lupus erythematosus (SLE) and was moved to a different hospital, after which we were unable to follow her clinical and laboratory data. From these results, USS-X5 could be considered to be a possible USS.

Family USS-Y

Patient

One female (USS-Y3) born in 1960.

Brief clinical data

USS-Y3 (*ADAMTS13* genotype: **p.G385E/p.R1206X**) was the last of three children to non-consanguineous parents. It is unclear whether this patient had a history of severe neonatal jaundice. However, during childhood she had an episode of thrombocytopenia and was diagnosed with ITP. She has a history of fresh whole blood transfusions, although the details are unclear. Since then, she had no remarkable changes. However, at 45 years of age, she suddenly developed thrombocytopenia and haemolytic anaemia, leading to a diagnosis of Evans syndrome. On this occasion, her physician noted many schistocytes on her blood film, and USS-Y3 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. An *ADAMTS13* gene analysis determined that she was a compound heterozygote with **p.G385E** (exon 10) from her father and **p.R1206X** (exon 26) from her mother.

Family USS-Z

Patient

One female (USS-Z3) born in 1971.

Brief clinical data

USS-Z3 (*ADAMTS13* genotype: **p.R193W/p.R193W**) was the last of three children to consanguineous parents (second cousins). Her clinical data were previously described [12]. Briefly, she became pregnant for the first time at 25 years of age, and at 12 weeks of gestation, she developed thrombocytopenia and was diagnosed with pregnancy-associated ITP. At 32 weeks of gestation, she had a live birth by caesarean section, and then developed overt TTP, which was treated with daily plasma exchange. This patient was referred to our laboratory in 1998, and USS-Z3 was confirmed to have a severe deficiency in ADAMTS13 activity in the absence of ADAMTS13 inhibitors. This patient did not receive prophylactic FFP infusions, and she had more than five TTP episodes between 1998 and 2005. Each episode was treated with 320 mL plasma infusions. She has been receiving prophylactic FFP infusions every 2 weeks.

Family USS-AA

Patient

One female (USS-AA3) born in 1987.

Brief clinical data

USS-AA3 (*ADAMTS13* genotype: not performed) was the first of two children born to non-consanguineous parents. She had neither an apparent history of severe neonatal jaundice nor thrombocytopenia during childhood. At 19 years of age, she suddenly developed petechiae, and her laboratory data indicated severe thrombocytopenia and haemolytic anaemia. Thus, her ADAMTS13 activity was examined and revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. Plasma exchange therapy was performed, and her platelet counts normalised. One month later, her ADAMTS13 activity and ADAMTS13 inhibitor status were re-tested and yielded the same results. In addition, her family members had the following ADAMTS13 activities: father (32%), mother (53%), and younger sister (46%). An *ADAMTS13* gene analysis was not performed in this family because permission was not obtained. In 2009, we determined that USS-AA3 had a normal platelet count (201×10^9 L^{-1}), but her ADAMTS13 activity was still very low (< 0.5% of normal) with no ADAMTS13 inhibitors. Since this point, we have been unable to obtain more up-dated information on this patient.

Family USS-BB

Patient

One male (USS-BB3) born in 1947.

Brief clinical data

USS-BB3 (*ADAMTS13* genotype: **p.R193W/p.R193W**) was the first of three children to consanguineous parents (first cousins). His younger sister died of 'purpura of unknown cause' at 23 years of age. It is unclear whether USS-BB3 experienced episodes of severe jaundice as a newborn or childhood thrombocytopenia. He was married and had three children. At 55 years of age, he developed overt TTP, which was successfully treated with plasma exchange. When he was 59 years old, he developed haematuria and was admitted to a nearby hospital, where an ADAMTS13 analysis showed that he had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis indicated that he was a homozygote with **p.R193W** (exon 6) (communication with Dr Toshi Imai, details will be reported by the physicians in charge).

Family USS-CC

Patient

One male (USS-CC5) born in 2004.

Brief clinical data

USS-CC5 (*ADAMTS13* genotype: **p.Q723K/p.R398C**) was the last of three children to non-consanguineous parents. Soon after birth, he developed Coombs-negative haemolytic anaemia and was treated with an exchange blood transfusion. At 7 months of age, he became infected with influenza A virus that aggravated his thrombocytopenia and haemolytic anaemia. At 32 months of age, he suddenly developed a transient disturbance in his ability to walk and converse. On this occasion, an ADAMTS13 analysis revealed that USS-CC5 had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis indicated that he was a compound heterozygote with **p.Q723K** (exon 18) from his father and **p.R398C** (exon 10) from his mother. Since he was diagnosed with USS, he has received prophylactic FFP infusions every 2 weeks.

Family USS-DD

Patient

One female (USS-DD5) born in 2007.

Brief clinical data

USS-DD5 (*ADAMTS13* genotype: **p.R268P/p.Y304C**) was born as the last of three children to non-consanguineous parents. One day after birth, the patient developed haematuria, petechiae, moderate jaundice, and thrombocytopenia, suggesting immune thrombocytopenia. A platelet transfusion was performed that subsequently aggravated her jaundice, which was ameliorated with albumin infusions and phototherapy from three directions. Therefore, an exchange blood transfusion was not performed. Her platelet counts were maintained around $60–100 \times 10^9$ L^{-1}, and at 15 days of age the physician infused FFP at a dose of 10 mL kg^{-1} due to suspected USS. This treatment markedly increased her platelet counts (written information from Dr Hitoshi Miyabayashi). One month after birth, ADAMTS13 analysis showed that the patient had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis determined that USS-DD5 was a compound heterozygote with **p.R268P** (exon 7) from her father and **p.Y304C** (exon 8) from her mother. The patient did not receive prophylactic FFP infusions.