considered important to administer anticoagulant treatment within 4 days after orthopaedic surgery.

These findings suggest that the appropriate cut-off values of D-dimer or SF is useful for the diagnosis of DVT after orthopaedic surgery.

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LETTER TO THE EDITOR

# Definite diagnosis in Japanese patients with protein C deficiency by identification of causative *PROC* mutations

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Protein C is a vitamin K-dependent plasma glycoprotein that functions as an important regulator of blood coagulation, and its deficiency is known to be a risk factor for thrombosis [1]. Congenital protein C deficiency is an autosomally inherited disorder, and has been classified into 2 types: a quantitative deficiency (type I), and qualitative deficiency (type II) [2]. Heterozygous patients with inherited protein C deficiency are mildly affected, and are either symptomatic (1 in 16,000 of the general population [3]) or asymptomatic (1 in 500 of the healthy population [4]). Thus, protein C deficiency in itself is thought to be a relatively mild risk factor for thromboembolism, and it is suggested that thrombosis-prone protein C deficient families might carry additional genetic factors that increase the risk, such as FV Leiden mutation and prothrombin 20210G > A mutation in Caucasian populations [5]. Severe congenital protein C deficiency is a much rarer disease, most often caused by a homozygous (or compound heterozygous) protein C gene (*PROC*) mutation(s). Some homozygous subjects develop purpura fulminans or skin necrosis and intravascular disseminated coagulation at birth [6–8], while heterozygous deficiency predisposes to venous thrombosis in adulthood. This clinical heterogeneity could reflect a variety of molecular mechanisms.

The prevalence of protein C deficiency in the general Japanese population was also estimated similarly to be approximately 1 in 500 [9]. In this study, we investigated the molecular defects of protein C deficiency in 6 Japanese patients, and defined respective causative mutations in the *PROC*. All patients were diagnosed as having protein C deficiency (Table 1) [10]. After informed consents were obtained, genomic DNAs were isolated from peripheral blood leukocytes of the patients and studied under approval of the study from the Ethics Committee of the Nagoya University School of Medicine. We amplified each of the 8 exons including the exon/intron boundaries of the *PROC* by polymerase chain reaction (PCR), and directly sequenced them as described previously [7]. We identified 6 distinct heterozygous *PROC* mutations in the 6 Japanese patients with protein C deficiency: 5 missense mutations (p.Arg42Ser [c.124C > A], p.Met406Ile [c.1218G > A], p.Cys147Tyr [c.439G > A], p.Arg211Trp [c.631C > T],

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**Table 1** Gene abnormalities and clinical features of patients with PC deficiency

| Case | Age | Sex | PC Ac (%) | PC Ag (%) | Mutation | Location | Predicted AA change | Thrombosis |
|---|---|---|---|---|---|---|---|---|
| 1 | 0 | F | <10 | ND | c.124C > A | Exon 3 | p.Arg42Ser | PF |
| | | | | | c.1218G > A | Exon 9 | p.Met406Ile | |
| 2 | 38 | M | 48 | 35 | c.1268delG | Exon 9 | p.Gly423ValfsX82 | PE |
| 3 | 30 | F | 52 | 42 | c.439G > A | Exon 7 | p.Cys147Tyr | DVT |
| 4 | 30 | F | 38 | 44 | c.631C > T | Exon 6 | p.Arg211Trp | DVT |
| 5 | 35 | F | 39 | 42 | c.1015G > A | Exon 9 | p.Val339Met | PVT |
| 6 | 0 | M | 10 | 5 | c.631C > T | Exon 6 | p.Arg211Trp | PF |
| | | | | | c.1268delG | Exn 9 | p.Gly423ValfsX82 | |

Nucleotide and amino acid numbers are described according to den Dunnen et al. [10]. (GenBank accession number: NT_022135)
*M* male, *F* female, *ND* not done, *PF* purpura fulminans, *PE* pulmonary embolism, *DVT* deep vein thrombosis, *PVT* portal vein thrombosis

and p.Val339Met [c.1015G > A]) and a G deletion (p.Gly423ValfsX82 [c.1268delG]). All mutations were previously reported in Japanese patients with protein C deficiency, except for c.439G > A which had been already reported in 2 Dutch families [11–13].

Two patients (Cases 1 and 6) with severe protein C deficiency, who both developed purpura fulminans shortly after birth, were found to be compound heterozygous for c.124C > A and c.1218G > A mutations, and c.631C > T and c.1268delG mutations, respectively. In Case 1, we could not obtained samples from the other family members including the parents, but we performed PCR including exons 3–9 of the *PROC* gene of the patient to define compound heterozygosity for the identified mutations (i.e., c.631C > T in exon 3 and c.1268delG in exon 9), as described previously [14]. Direct sequencing data from the subclones of the PCR fragments showed c.631C > T or c.1268delG mutation independently, and no subclone with both mutations was obtained, indicating that the respective *PROC* mutations might have been inherited from his parents separately (data not shown). In Case 6, we performed PCR-RFLP analyses as described in previously [15], to examine inheritance of the mutations (c.631C > T and c.1268delG) identified in the patient. PCR-*Sac* II RFLP analysis to detect the c.631C > T mutation in the *PROC* showed that the patient and his mother had a mutant allele, but his father did not (Fig. 1a). We also employed mismatch PCR-*Eco*N I RFLP analysis to detect the c.1268delG mutation in the *PROC*, showing that the patient and his father had a mutant allele, but his mother did not (Fig. 1b). These data indicated that the patient inherited the c.631C > T mutation from his mother and the c.1268delG mutation from his father, suggesting that the patient could be compound heterozygous. These observations were consistence with previous reports that severe protein C deficiency caused by a homozygous (or compound heterozygous) *PROC* mutation(s) frequently led to infantile purpura fulminans [2]. Diagnosing homozygote infants with protein C deficiency depends upon the appropriate clinical picture, a protein C level that is essentially unmeasurable, and the confirmation of heterozygous parents. To confirm the diagnosis of this rare disorder, it may be necessary to ascertain the presence of *PROC* mutations in the family members including the parents.

Numerous cases of hereditary protein C deficiency have been reported, showing that individuals with protein C deficiency tend to have an increased risk of thromboembolism [2], and over two hundred different *PROC* mutations have been identified so far. It was reported that five recurrent defects (c.541T > G, c.631C > T, c.1015G > A, c.1218G > A, and c.1268delG, which were founder or hot spot mutations) may account for 49% of Japanese families with protein C deficiency [11]. Consistently, we found in this study that 5 out of the 6 patients had either one or two of these recurrent mutations. Especially, one (Case 6) of the compound heterozygous patients with severe protein C deficiency had two of the Japanese recurrent *PROC* mutations, and the other (Case 1) had one of the recurrent mutations and the previously reported *PROC* mutation in Japanese. These data again suggest that it might be necessary to survey *PROC* mutations including Japanese recurrent *PROC* mutations in Japanese patients with such severe protein C deficiency in order to make an accurate diagnosis and start appropriate treatment immediately.

In summary, we investigated molecular defects of protein C deficiency in 6 Japanese patients, and defined respective causative mutations in the *PROC*, most of which were recurrent *PROC* mutations in the Japanese population.

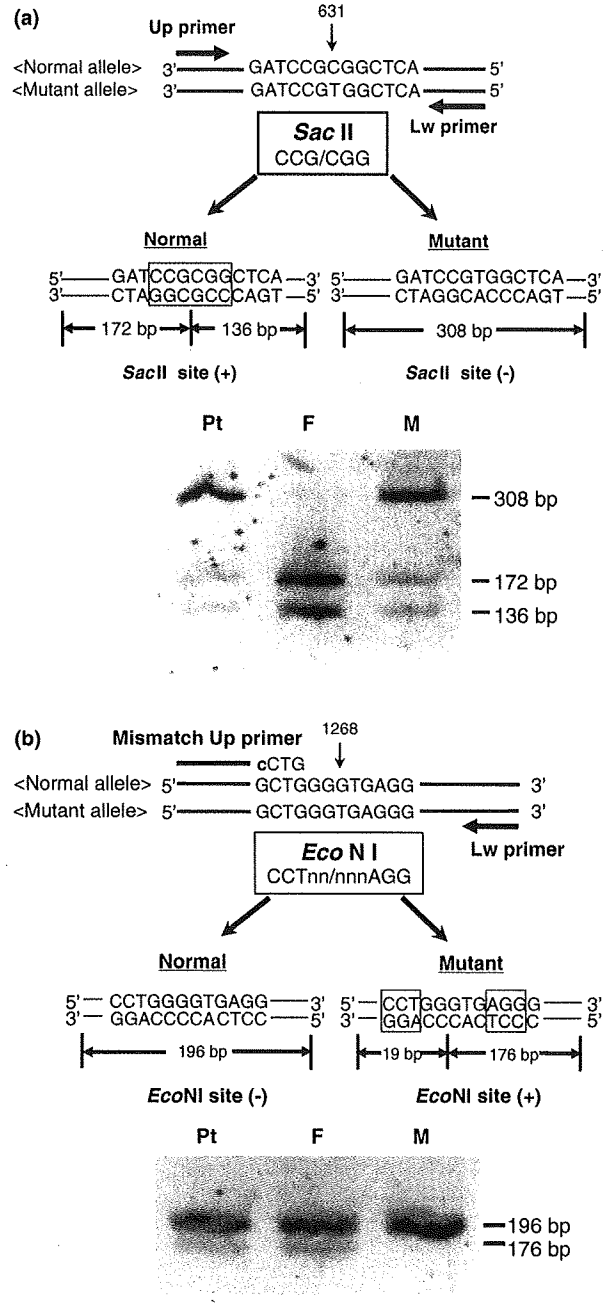


**Fig. 1** PCR-RFLP analyses of the family of Case 6. **a** Strategy (*upper*) and result (*lower*) of PCR-*Sac* II RFLP analysis to detect c.631C > T mutation in *PROC*. Pt: patient, F: father, M: mother. **b** Strategy (*upper*) and result (*lower*) of mismatch PCR-*Eco*N I RFLP analysis to detect c.1268delG mutation in *PROC*. Pt: patient, F: father, M: mother

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# A novel missense mutation causing abnormal LMAN1 in a Japanese patient with combined deficiency of factor V and factor VIII

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**Combined deficiency of coagulation factor V (FV) and factor VIII (FVIII) (F5F8D) is an inherited bleeding disorder characterized by a reduction in plasma concentrations of FV and FVIII. F5F8D is genetically linked to mutations in either *LMAN1* or *MCFD2*. Here, we investigated the molecular basis of F5F8D in a Japanese patient, and identified a novel missense mutation (p.Trp67Ser, c.200G>C) in the *LMAN1*, but no mutation in the *MCFD2*. The amount of LMAN1 in Epstein-Barr virus-immortalized lymphoblasts from the patient was found to be almost the same as that in cells from a normal individual. Interestingly, an anti-MCFD2 antibody did not co-immunoprecipitate the mutant LMAN1 with MCFD2 in lymphoblasts from the patient, suggesting the affinity of MCFD2 for the mutant LMAN1 is weak or abolished by the binding of the anti-MCFD2 antibody. In addition, a Myc/6×His-tagged recombinant form of wild-type LMAN1 could bind to D-mannose, but that of the mutant could not. The p.Trp67Ser mutation was located in the carbohydrate recognition domain (CRD), which is thought to participate in the selective binding of LMAN1 to the D-mannose of glycoproteins as well as the EF-motif of MCFD2. Taken together, it was suggested that the p.Trp67Ser mutation might affect the molecular chaperone function of LMAN1, impairing affinity for D-mannose as well as for MCFD2, which may be responsible for F5F8D in the patient. This is the first report of F5F8D caused by a qualitative defect of LMAN1 due to a missense mutation in *LMAN1*. Am. J. Hematol. 84:738–742, 2009.** © 2009 Wiley-Liss, Inc.

## Introduction

Coagulation factor V (FV) and factor VIII (FVIII) are both essential in the blood coagulation cascade, as cofactors for the proteases factor X and factor IX, respectively. Combined deficiency of FV and FVIII (F5F8D) is an autosomal recessive bleeding disorder first described by Oeri et al. in 1954 [1], and a distinct clinical entity from chance co-inheritance of hemophilia A (FVIII deficiency) and parahemophilia (FV deficiency). F5F8D is extremely rare (1:2,000,000) in the general population [2], and characterized by a mild-to-moderate bleeding tendency manifested after surgical trauma, abortion, and delivery. Menorrhagia is also common, but hematuria and gastrointestinal bleeding are infrequent, and hemarthrosis is rare [3]. Generally, patients with F5F8D show plasma levels of FV and FVIII in the range of 5–30% of normal [4].

Positional cloning has identified two genes, *LMAN1* (lectin, mannose-binding, 1; also known as ERGIC-53) and *MCFD2* (multiple coagulation factor deficiency gene 2), associated with F5F8D [4,5] LMAN1 is a Type-1 transmembrane protein that cycles between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment (ERGIC) [6,7]. It contains a mannose-specific carbohydrate recognition domain (CRD) on the ER luminal side, and ER exit and retrieval motifs on the cytoplasmic side [8]. MCFD2 has an EF-hand domain that interacts with LMAN1 in a $Ca^{2+}$-dependent manner [5]. The LMAN1-MCFD2 protein complex functions as a cargo receptor that facilitates the transport of FV and FVIII from the ER to the Golgi apparatus [5,9].

Extensive genetic analyses of F5F8D patients have identified many causative mutations in *LMAN1* and *MCFD2*, which may account for nearly all cases, with about 70% of familial cases of F5F8D attributable to *LMAN1* mutations and about 30% to *MCFD2* mutations [10]. Most of these mutations, especially in *LMAN1*, were nonsense, frame shift, splicing defect, or missense mutations, resulting in null alleles.

In this study, we examined the molecular basis of F5F8D in a Japanese patient, and identified a novel homozygous missense mutation in *LMAN1* that led to a functionally abnormal LMAN1 causing the F5F8D.

## Results

### Case report

The patient was a 32-year-old female suffering from bleeding after the third delivery, having had similar bleeding

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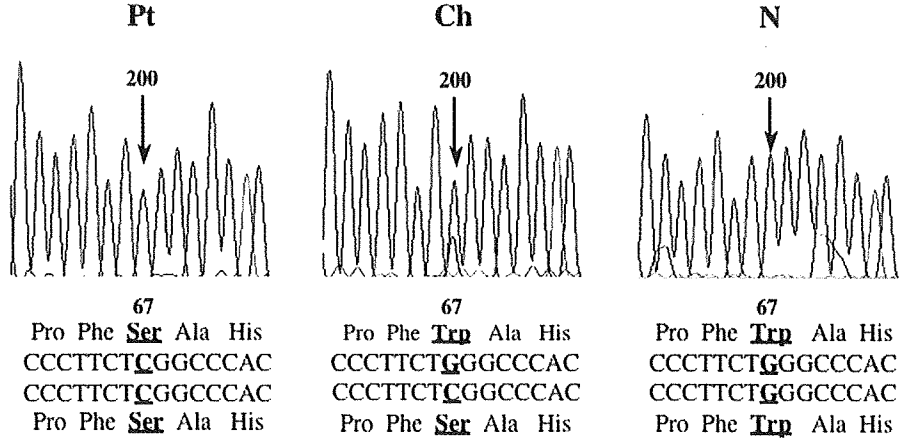


Figure 1. Sequence analysis of *LMAN1*. Nucleotides and predicted amino acids sequences surrounding the mutation of exon 1 in the *LMAN1* gene. Arrows indicate the site of mutation. The mutation was a G-to-C transversion at nucleotide 200, leading to a substitution of TGG (Trp) at codon 67 with TCG (Ser). Pt, patient; Ch, child; N, normal individual.

episodes in her previous two deliveries. Her platelet count, FDP-E, and liver function tests were normal, but both her PT (19.7s/control 12.0s) and APTT (72.5s/control 43.6s) were prolonged. She was diagnosed with F5F8D, because her FV and FVIII coagulant activities were 14% and 19%, respectively, and the activities of the other coagulation factors were normal (fibrinogen, 275 mg/ml; FII, 78%; FVII, 96%; FX, 83%; FIX, 110%; FXI, 70%; FXII, 84%; PK, 70%; HMWK, 104%). To investigate the molecular basis of her F5F8D, she was referred to the Nagoya University hospital.

### Mutational analysis in the F5F8D patient

We analyzed coding regions and intron-exon boundaries of *LMAN1* and *MCFD2* of the patient with F5F8D by PCR-mediated direct sequencing, and identified a novel missense mutation in *LMAN1*, but no mutation in *MCFD2*. The mutation, found in exon 1 of *LMAN1*, was a G-to-C transversion at nucleotide 200 (c.200G>C), leading to the substitution of TGG (Trp) at codon 67 with TCG (Ser) (p.Trp67Ser) (Fig. 1). The patient turned out to be homozygous for the c.200G>C mutation, as confirmed by a *Apa*I PCR-restriction fragment length polymorphism (RFLP) analysis (data not shown). A large deletion of *LMAN1* in the other allele of the patient was excluded because of heterozygosity for a SNP (refSNP ID: rs11354119) in intron 6 of *LMAN1* (data not shown). Her child was also found to be heterozygous for this mutation by DNA sequencing as well as the *Apa*I-RFLP analysis.

### Western blot analysis and ELISA for LMAN1 protein

To determine the expression levels of LMAN1 in vivo, we prepared Epstein-Barr virus (EBV)-immortalized lymphoblast lines derived from the patient, her child, and a healthy volunteer, and measured LMAN1 antigens by Western blot analysis as well as by enzyme-linked immunosorbent assay (ELISA). LMAN1 proteins of the expected molecular size (53 kDa) were detected in all samples by Western blotting (Fig. 2A), and no difference in relative absorbance was found among the samples by ELISA for LMAN1 (Fig. 2B). These results indicated that the amounts of mutant LMAN1 expressed in lymphoblasts from the patient were same as those of wild-type LMAN1 in normal control lymphoblasts.

### Immunoprecipitation-Western blot analysis

To examine the relation between LMAN1 and MCFD2 in the EBV-immortalized lymphoblasts, we performed immunoprecipitation (IP)-Western blotting using anti-MCFD2 and

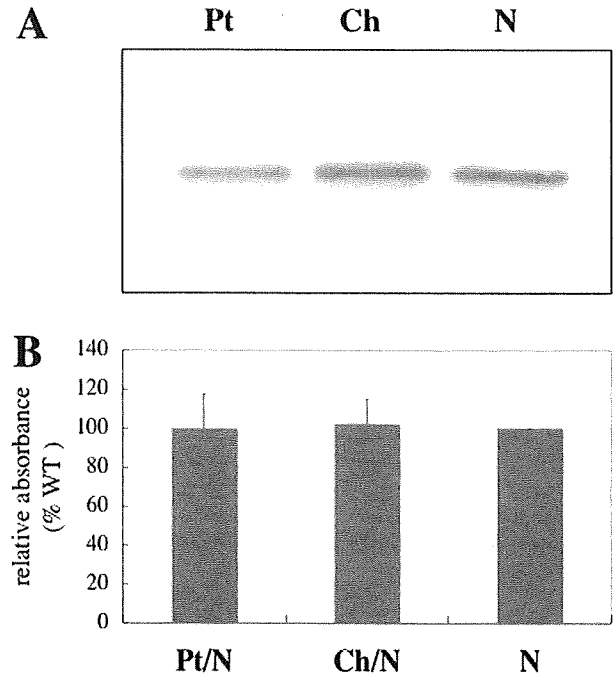


Figure 2. Quantification of LMAN1 proteins in lymphoblasts. A: Western Blot analysis of LMAN1 in lymphoblasts from the patient, her child, and a normal individual. LMAN1 protein of the expected size, 53 kDa, was detected in all samples. B: LMAN1 levels in lymphoblasts determined by ELISA. We calculated the absorbance of the sample relative to that of a normal sample (Mean ± SD, $n$ = 3). Pt, patient; Ch, child; N, normal individual.

anti-LMAN1 antibodies, with 10 mM $Ca^{2+}$ or 10 mM EDTA present in the lysis buffer. In lymphoblasts from the normal individual, we observed that the anti-MCFD2 antibody co-immunoprecipitated LMAN1 with MCFD2 in the presence of 10 mM $Ca^{2+}$, but not 10 mM EDTA (Fig. 3A). In lymphoblasts from the patient, however, the anti-MCFD2 antibody did not co-immunoprecipitate mutant LMAN1 at all, even in the presence of 10 mM $Ca^{2+}$. In lymphoblasts from the child carrying the heterozygous mutation, the signal for LMAN1 co-immunoprecipitated with the anti-MCFD2 antibody was slightly weaker than that in lymphoblasts from the normal control in the presence of 10 mM $Ca^{2+}$. These results indicated that the mutant 67Ser-LMAN1 associated with MCFD2 to a much lesser extent than wild-type LMAN1 in lymphoblasts.

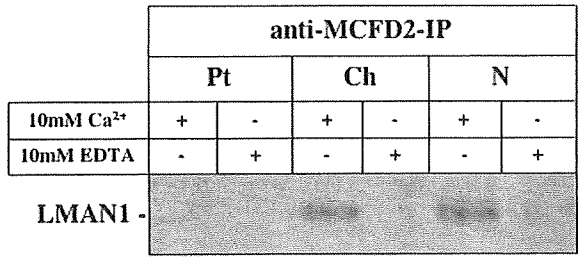


Figure 3. IP-Western blot analysis of LMAN1/MCFD2 complex samples immunoprecipitated with a goat anti-MCFD2 antibody from lymphoblast lysates, with 10 mM $Ca^{2+}$ or 10 mM EDTA present in the lysis buffer, were analyzed by Western blotting for LMAN1 using a chicken anti-LMAN1 antibody. Pt, patient; Ch, child; N, normal individual.

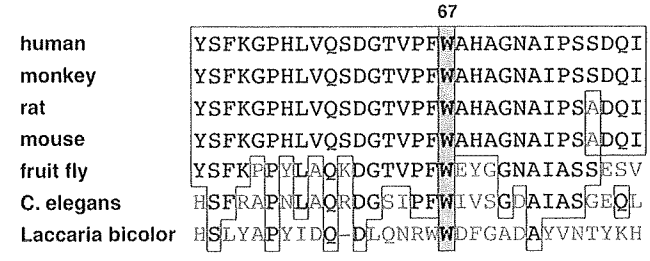


Figure 5. Comparison of amino acid sequences of human, monkey, rat, mouse, fruit fly, *C. elegans*, and *Laccaria bicolor* LMAN1. Open boxes denote amino acids that do not differ between human LMAN1 and LMAN1 from other species. 67 Trp (W in bold in the shaded box) is conserved in all species.

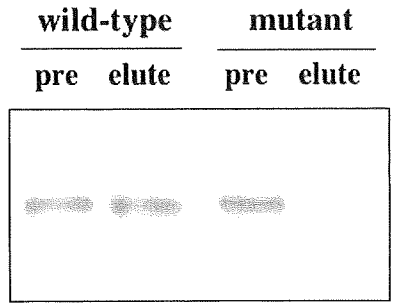


Figure 4. Mannnose-binding assay. A wild-type (67 Trp) or mutant (67 Ser) recombinant LMAN1 bearing myc/6×His tags were expressed in COS1 cells and purified by Ni-NTA agarose. The samples were then incubated with mannose agarose, eluted with 0.2 M D-mannnose, immunoprecipitated with an anti-c-myc antibody, and detected by Western blotting with an anti-LMAN1 antibody. pre, samples pre-treated with mannose agarose; elute, samples eluted from mannose agarose.

### Mannose-binding assay for recombinant Myc/6×His-LMAN1

To test the ability to bind to mannose agarose, we prepared wild-type and mutant recombinant LMAN1 bearing myc/6×His tags. The recombinant myc/6×His-LMAN1 eluted from mannose agarose with 0.2 M D-mannose were immunoprecipitated with an anti-c-myc antibody, and detected by Western blotting with an anti-LMAN1 antibody. We demonstrated that the wild-type myc/6×His LMAN1 bound to mannose agarose, whereas the mutant myc/6×His LMAN1 did not (Fig. 4). We also observed that the wild-type recombinant LMAN1 bound to the mannose in a $Ca^{2+}$-dependent manner as reported previously [11] (data not shown).

## Discussion

F5F8D is a very rare congenital bleeding disorder and recent extensive genetic analyses of F5F8D patients have identified many causative mutations in the *LMAN1* and *MCFD2* genes. We, here, investigated the molecular basis of F5F8D in a Japanese patient, and identified a novel missense mutation (p.Trp67Ser, c.200G>C) in the *LMAN1*, but no mutation in the *MCFD2*.

To date, at least 32 *LMAN1* mutations have been reported throughout all 13 exons of the gene, most of which are either nonsense or frameshift mutations leading to the complete absence of a functional LMAN1 protein [12–14]. So far, only two missense mutations have been reported in *LMAN1*, p.Met1Thr, and p.Cys475Arg, which resulted in little or no LMAN1 protein in the cells [13,15]. In this report, we showed that lymphoblasts from a patient with a homozygous p.Trp67Ser mutation possessed a variant LMAN1 protein in the same amount as LMAN1 in lymphoblasts from a normal individual. These results implied that the variant 67Ser-LMAN1 might be functionally abnormal as a cargo receptor in a complex with MCFD2 for trafficking FV and FVIII in this F5F8D patient.

LMAN1 is thought to form a $Ca^{2+}$-dependent complex with MCFD2 in the ER lumen at a stoichiometry of 1:1. Direct molecular interaction between LMAN1 and MCFD2 can occur in vivo, because these two proteins can be co-immunoprecipitated in a complex from cells [5,9]. We examined the interaction between LMAN1 and MCFD2 in lymphoblasts by IP-Western blot analysis using anti-MCFD2 antibody, and found that the variant 67Ser-LMAN1 of the patient impaired $Ca^{2+}$-dependent association with MCFD2. The CRD of LMAN1 would contain the MCFD2-binding site, because the LMAN1-MCFD2 complex was reconstituted in vitro using the purified CRD of LMAN1 and MCFD2 [16]. In addition, the alignment of LMAN1 and related sequences from different organisms within the animal kingdom revealed a high degree of sequence identity including 67 Trp (Fig. 5), which located in the CRD of LMAN1 is one of the tryptophans forming a hydrophobic ladder running through the hydrophobic core of the protein [17]. In the crystal structure study of the CRD, Velloso et al. showed that 67 Trp locates in the first strand (β2) of the major β-sheet consisting an edge of CRD, and concluded that the major β-sheet (β2, β5, β14, β7–β10) curved giving rise to a concave surface, which is the putative ligand binding site (Fig. 6) [17]. Therefore, it is likely that the p.Trp67Ser missense mutation would affect the conformation of the CRD in LMAN1 leading to a change in affinity for MCFD2.

It was reported that the recombinant CRD of LMAN1 could bind to sugars in a $Ca^{2+}$-dependent manner, and this was enhanced by its interaction with MCFD2 [16]. In the present study, we demonstrated that the recombinant wild-type LMAN1 could bind to mannose in the presence of $Ca^{2+}$ ions in vitro, even in the absence of MCFD2. In contrast, the mutant 67Ser-LMAN1 lost its sugar-binding ability, suggesting that the p.Trp67Ser missense mutation in the CRD would abolish the binding to mannose, possibly due to a conformational change of the CRD in LMAN1.

It was likely that the p.Trp67Ser mutation in LMAN1 abolished its sugar-binding ability and impaired its interaction with MCFD2, resulting in the reduced secretion of factors V and VIII in this F5F8D patient. To our knowledge, this is the first report of F5F8D caused by a functionally abnormal LMAN1 due to a missense mutation in *LMAN1*.

## Materials and Methods

*Patients and samples.* The study was approved by the Ethics Committee of the Nagoya University School of Medicine. After obtaining informed consents, blood samples were collected from the patient and one of her children, but other family members did not participate in this study. Genomic DNA was isolated from peripheral blood leukocytes

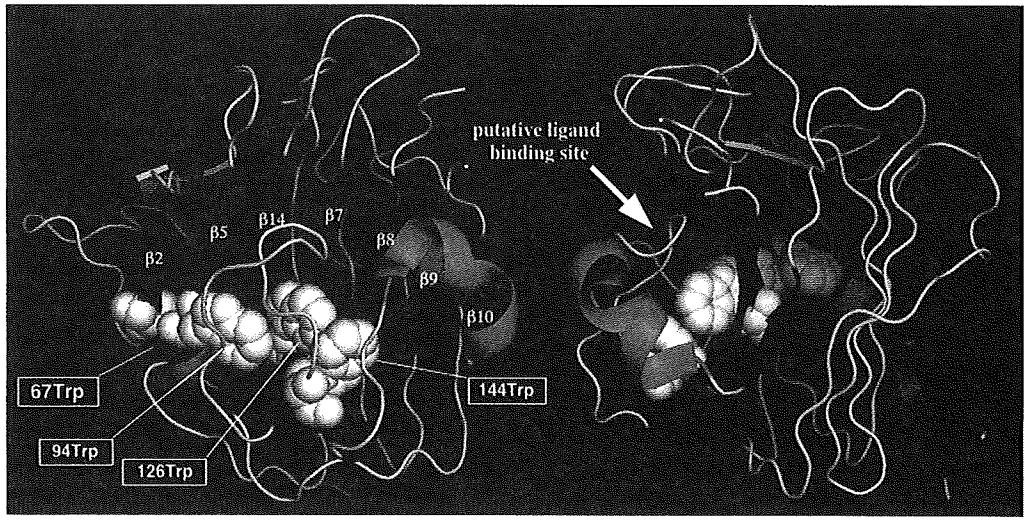


Figure 6. Structural model of carbohydrate recognition domain of LMAN1. Overall structure of LMAN1 monomer was maid by MacPyMOL according to the data form PDB: 1GV9 [17]. 67 Trp is one of the tryptophans (67 Trp, 94 Trp, 126 Trp, and 144W) forming a hydrophobic ladder running through the hydrophobic core of the protein. 67 Trp locates in the first strand (β2) of the major β-sheet consisting an edge of CRD, and the major β-sheets (β2, β5, β14, β7–β10) are though to curve giving rise to a concave surface, which is the putative ligand binding site [17].

according to a standard procedure. We also established Epstein-Barr virus (EBV)-immortalized lymphoblast lines from the patient, her children, and a control individual.

*DNA sequencing of LMAN1 and MCFD2.* All coding exons and intron-exon junctions of *LMAN1* and *MCFD2* were amplified from the genomic DNA by a polymerase chain reaction (PCR) as described previously [18]. The PCR products were directly sequenced using a Big-Dye Terminator Cycle Sequencing kit and a 310 Genetic Analyzer (Applied Biosystems. Foster City, CA).

*Western blot analysis.* EBV-immortalized lymphoblasts derived from the patient, her child, and a healthy control were dissolved in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$, and 1% Triton X-100), the lysate were cleared by centrifugation at 1,500*g*, and protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA). Equal amounts of cell lysate (1.5 μg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. LMAN1 was detected by immunostaining with a chicken anti-LMAN1 IgY antibody (GenWay Biotech, San Diego, CA) and a rabbit anti-chicken IgY conjugated with horseradish peroxidase (GenWay Biotech), using an ECL PLUS Western blotting detection system (Amersham Biosciences, Piscataway, NJ).

*Enzyme-linked immunosorbent assay for the measurement of LMAN1.* We also measured antigen levels of LMAN1 in equal amounts of lysate (15μg) with an enzyme-linked immunosorbent assay (ELISA) as described previously [19]. Briefly, a polyclonal chicken anti-LMAN1 antibody was used for capturing, while amplification and detection of the signals were achieved with a biotinylated polyclonal chicken anti-LMAN1 antibody and avidin peroxidase using an ECL Protein Biotinylation Module kit (Amersham Biosciences). We calculated the absorbance of the sample relative to that of a normal sample (Mean ± SD, $n = 3$).

*Immunoprecipitation-Western blot analysis.* Equal amounts of lymphoblast lysate (250 μg) in the presence of 10 mM $Ca^{2+}$ or 10 mM EDTA in the lysis buffer were immunoprecipitated with a goat anti-MCFD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using protein G PLUS-Agarose (Santa Cruz Biotechnology). The precipitated samples were then separated by 10% SDS-PAGE and transferred to PVDF membrane. The signals were detected as described earlier for LMAN1.

*Construction of expression vectors for wild-type and mutant LMAN1s bearing an N-terminal c-myc epitope tag and a C-terminal 6×His tag.* A full-length human LMAN1 cDNA was amplified by PCR from a human liver cDNA library (Clonetech, Mountain View, CA), and cloned into the pCI vector (Promega, Madison, WI). To allow for the simplified detection and purification of the recombinant LMAN1, we engineered a myc/6×His-tagged LMAN1 expression vector as described previously [11]. First, a c-myc epitope was introduced at the N-terminus of mature LMAN1 by recombinant PCR using mutagenesis primers, 5′-CAGATCC TCTTCTGAGATGAGTTTTTGTTCGCCCCGGACGAAGCGAC (forward: mutated nucleotides are underlined) and 5′-CTCATCTCAGAAGAGGA TCTGGACGGCGTGGGAGGAGA (reverse), as described elsewhere [20]. Second, a 6×His tag was introduced at the C-terminus, replacing the targeting signal (KKFF) of LMAN1, by substitution with a PCR fragment using a mutagenesis reverse primer, 5′-CCTCAATGGTGA TGGTGATGATGGGCAGCTGCTTCTTGCT. Subsequently, we introduced a c.200G>C mutation into the myc/6×His-tagged LMAN1 by substitution with a PCR product using a forward mutagenesis primer, 5′-GCACCTGGTGCAGAGCGACGGGACCGTGCCCTTCTCGGCC. The presence of the desired mutations and the absence of a second mutation in the expression vectors were confirmed by DNA sequencing.

*Transient expression of recombinant LMAN1 in COS1 cells.* COS-1 cells were grown in DMEM supplemented with 10% fetal calf serum. The cells were cultured in 100-mm dishes until about 50% confluent, and then transiently transfected with 20 μg of the expression plasmid vectors using the calcium phosphate method as described previously [21]. After 48 hr of incubation, the cells were harvested and dissolved in the lysis buffer as described earlier. The cell lysates were then centrifuged at 1,500*g* for 10 min and cleared supernatants were used for subsequent mannose-binding experiments.

*Mannose-binding assay for recombinant LMAN1.* The cleared cell lysates containing wild-type or mutant Myc/6×His LMAN1 were incubated with Ni-NTA agarose (Invitrogen, Carlsbad, CA) under constant agitation for 1 hr at 4°C. After washing with binding buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.15% Triton X-100), bound proteins were eluted with 0.25 M imidazole and dialyzed against the binding buffer. The protein concentrations in the dialyzed samples were quantified using the Bio-Rad Protein Assay Kit. The samples (4 μg each of total protein) were incubated with 200 μl of mannose agarose (EY Laboratories, San Mateo, CA) under constant agitation overnight at 4°C. After washing, bound recombinant LMAN1 was eluted with 200 μl of 0.2 M D-mannnose (Sigma-Aldrich, St. Louis, MO) in the binding buffer, and then immunoprecipitated with anti-c-myc antibody (Santa Cruz Biotechnology) coupled to Protein G PLUS-Agarose. Subsequently, the precipitates and the pre-mannnose agarose samples (0.12 μg of total protein for the wild-type and 0.4 μg for the mutant) were analyzed by 10% SDS-PAGE and Western blotting using a polyclonal chicken anti-LMAN1 antibody as described earlier.

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Regular Article

# Impaired secretion of carboxyl-terminal truncated factor VII due to an *F7* nonsense mutation associated with FVII deficiency

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ABSTRACT

*Introduction:* Factor VII (FVII) is a vitamin K-dependent glycoprotein secreted into the blood circulation from hepatic cells. We investigated the molecular basis of the congenital FVII deficiency found in a Japanese patient.
*Materials and Methods:* We analyzed the *F7* gene of the patient, who was diagnosed with a FVII deficiency at pregnancy. We expressed a carboxyl-terminal truncated FVII (Arg462X FVII) corresponding to the identified mutation in CHO-K1 cells. To study roles of the carboxyl-terminus in the secretion of FVII, we also expressed a series of recombinant FVIIs deleted of limited numbers of carboxyl-terminal amino acids (462Arg-466Pro).
*Results:* We identified a nonsense mutation (c.1384C>T: p.Arg462X) in *F7*, leading to a lack of five amino acids in the carboxyl-terminus. In expression experiments, Arg462X FVII was undetectable not only by Western blotting, but also by ELISA. A Western blot analysis of the truncated FVIIs revealed that all mutants were expressed in the cells the same as the wild type, but were secreted into the culture medium in lesser amounts than the wild type depending on the length of the deletion, which was confirmed by ELISA. Arg462X FVII did not colocalize with the Golgi on immunofluorescence staining, suggesting that it might be retained in the ER and degraded in the cell.
*Conclusion:* The carboxyl-terminal amino acids of FVII play an important role in its secretion, and the p.Arg462X mutation was likely to have caused the FVII deficiency in this patient.


## Introduction

Factor VII (FVII), a vitamin K-dependent plasma glycoprotein, is synthesized in the liver and secreted into the blood as a single-chain zymogen. Mature plasma FVII is composed of 406 amino acid residues with an apparent molecular weight of 50,000 and is present at a concentration of 500 ng/ml in normal plasma [1,2]. Upon vascular injury and in the presence of calcium, FVII forms a one-to-one stoichiometric complex with its cell surface receptor and cofactor, tissue factor (TF). Once in a complex with TF, FVII is rapidly cleaved to its active form, FVIIa, and converts zymogen factor IX and factor X into active enzymes [3,4]. The formation of an active complex between TF and FVIIa is widely thought to be the primary stimulus for blood coagulation.

The gene for FVII has been cloned and completely sequenced [5]. It is located on chromosome 13 (13q34), just 2.8 kb upstream of the factor X gene, and comprises 9 exons spanning about 12.5 kb. The FVII gene shares a common intron-exon structure with the genes of several other vitamin K-dependent blood coagulation proteins further suggesting they evolved through gene duplication events.

Hereditary FVII deficiency is a rare autosomal recessive bleeding disorder with variable clinical expression [6], and has an estimated incidence of 1 per 500,000 in the general population [7]. The hemorrhagic diathesis in affected patients can be highly variable, and does not necessarily correlate with plasma FVII activity levels [8]. Considering the poor relationship between FVII activity and the bleeding tendency, a molecular diagnosis is helpful in unsolved cases or in cases in which the pattern of inheritance is not clear. To date, extensive genetic analyses of patients with FVII deficiency have identified many causative mutations in the FVII gene (*F7*) (HMGD; http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F7).

*Abbreviations:* FVII, factor VII; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; TF, tissue factor; PT, Prothrombin time; APTT, activated partial thromboplastin time; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PVDF, polyvinylidene difluoride; PDI, protein disulfide isomerase; FITC, fluorescein isothiocyanate; FIX, factor IX; PC, protein C.

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In the present study, we identified a nonsense mutation in the *F7* gene of a Japanese patient deficient in FVII, and investigated the molecular consequence of the mutation by conducting *in vitro* expression experiments.

## Materials and Methods

### *Preparation of samples and coagulation tests*

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine. A blood sample was taken from the patient and collected in a 1/10 volume of 3.2% (w/v) trisodium citrate, after informed consent was obtained. The plasma was then separated by centrifugation at 2000×*g* for 20 min, and aliquots were stored at -70 °C until use. Genomic DNA was isolated from the peripheral blood leukocytes by phenol extraction as described previously [9]. Prothrombin time (PT), activated partial thromboplastin time (APTT), and FVII procoagulant activity (FVII: C) were measured as described [10].

### *Direct sequencing of DNA*

All exons and splice junctions of the *F7* gene were amplified by the polymerase chain reaction (PCR) using the gene-specific primers listed in Table 1. The amplification and Cycle sequencing of all PCR products were performed as described previously [11].

### *PCR-restriction fragment length polymorphism (RFLP) analysis*

To confirm the mutation (c.1384C>T) identified in *F7* of the patient, PCR-restriction fragment length polymorphism (RFLP) was performed. Since the mutation did not create any available restriction enzyme sites, we used a mismatched antisense primer (5'-CTGCTGG GCTAGGGAAATGGtGaTC-3'; substituted bases are underlined small letters) to introduce a *Bcl*I restriction site into the products amplified from the mutant allele. The PCR products were then treated with *Bcl*I and electrophoresed on a 2.0% agarose gel.

### *Construction of recombinant FVII expression vectors*

We prepared a wild-type FVII expression vector (pcDNA-FVIIWT) by introducing a FVII cDNA into the vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) as described previously [10]. To prepare expression vectors producing a series of recombinant FVIIs with limited deletions of the carboxyl-terminal sequence (Arg462X, Ala463X, Pro464X, Phe465X and Pro466X), we performed site-directed mutagenesis by PCR using the primers listed in Table 2. The PCR-amplified DNA fragments were isolated as *Sac*II/*Apa*I fragments, and inserted into pcDNA-FVIIWT. The sequences of all FVII expression vectors were confirmed by direct sequencing.

**Table 1**
Oligonucleotide primers for amplifying the FVII gene.

| Exon | Strand | Oligonucleotide Sequence | Product (bp) | Annealing (°C) |
|---|---|---|---|---|
| 1a | S | 5'-CACACCTTAACACCTGCACGCT-3' | 392 | 64 |
| | AS | 5'-GCCCACTGCCCTTCCACC-3' | | |
| 1b | S | 5'-GGGGTGGGCTGTGAGGGA-3' | 225 | 68 |
| | AS | 5'-ATGGGAGGGGAAGGAGGTGA-3' | | |
| 2 | S | 5'-CAGCGCCGCTCCCCTCCTC-3' | 339 | 65 |
| | AS | 5'-TTCACCGCCGCCGTGCAG-3' | | |
| 3,4 | S | 5'-GGTGTGTCCAGTGCTTACCGTT-3' | 344 | 69-64* |
| | AS | 5'-GGCCACCTCCACCAGCTC-3' | | |
| 5 | S | 5'-ACAGCTCATGCCACCTTCC-3' | 350 | 62 |
| | AS | 5'-CCAGTCCCACCCGTCTTT-3' | | |
| 6 | S | 5'-GGCCTCTCAGAGGATGGGT-3' | 284 | 62 |
| | AS | 5'-TCCCTTCCAGAGCTGTTGTT-3' | | |
| 7 | S | 5'-GGCGAGTCATCAGAGAAACAA-3' | 250 | 60 |
| | AS | 5'-GTCGGACAGGGAAGAGTGG-3' | | |
| 8-1 | S | 5'-GTGGCAGGTGGTGGAAAG-3' | 394 | 64 |
| | AS | 5'-TTCGTGATATTTGGGGAGT-3' | | |
| 8-2 | S | 5'-CTGGAGCTCATGGTCCTCAA-3' | 393 | 62 |
| | AS | 5'-CCAGGACAGTTCGACGCAG-3' | | |

*69-64: Annealing temperature of touchdown PCR.

**Table 2**
Oligonucleotide primers for the construction of recombinant FVII cDNA.

| | Mutants | Oligonucleotide Sequences |
|---|---|---|
| Sense | | 5'-CCTGATCAACACCATCTGGGTGGTCT-3' |
| Antisense | pcDNA-Arg462XFVII | 5'-GCTGGGCccGGGAAATGGGGCTCaCA-3' |
| | pcDNA-Ala463XFVII | 5'-GGGCTGCTGGGCccGGGAAATGGtcaTCGCA-3' |
| | pcDNA-Pro464XFVII | 5'-GCTGCTGGGCccGGGAAATcaGGCTCGCA-3' |
| | pcDNA-Phe465XFVII | 5'-TGCTGGGCccGGGtcATGGGGCTCGCA-3' |
| | pcDNA-Pro466XFVII | 5'-GCTGGGCcctcaAAATGGGGCTCGCAGG-3' |

Small letters are mismatched nucleotides.
Single- and double-underlines indicate the *Apa*I site and stop codon sequence created by the nucleotide substitution, respectively.

### *Cell culture and recombinant FVII expressions*

Chinese hamster ovary (CHO)-K1 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 10 mg/ml of vitamin $K_1$ (Isei, Yamagata, Japan) at 37 °C in a humidified atmosphere containing 5% $CO_2$. The transient expression of recombinant FVIIs was achieved by transfection with 20 μg of each expression vector in 100 mm culture plates using the Ca-phosphate method as described previously [12]. The cells were incubated for 24 hrs in serum-free medium containing 10 mg/ml of vitamin $K_1$. The cell lysate dissolved in Reporter Lysis Buffer (Promega, Madison, WI) and the culture medium concentrated with a Centrisart I kit (Sartorius, Goettingen, Germany) were subjected to Western blotting.

We also established stable transformants expressing recombinant FVIIs as described previously [13]. The media were collected from confluent plates grown for 24 hrs in serum-free medium containing 5 mg/ml of vitamin $K_1$ and centrifuged at 12,000×g for 20 min at 4 °C, and the supernatants were used for ELISA as described below. The protein concentrations of the samples were determined with a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA), using bovine serum albumin as a standard.

### *Western Blotting Analysis*

The samples (15 μg) of cell lysates and culture medium containing the respective recombinant FVIIs were separated by SDS-PAGE (10%), and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ). FVII proteins were detected by immunostaining with a polyclonal rabbit anti-FVII (Nordic Immunological laboratories, Tilburg, The Netherlands) and a goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Cell Signaling Technology Inc., Danvers, MA), using an ECL PLUS Western blotting detection system (Amersham Biosciences).

### *Enzyme-linked immunosorbent assay (ELISA)*

To measure concentrations of the recombinant FVIIs in the culture media, an enzyme-linked immunosorbent assay (ELISA) was performed as described previously [14]. A monoclonal anti-FVII antibody (Innovative Research Inc., Minneapolis, MN) was used for capture and a peroxidase-conjugated polyclonal anti-FVII antibody (Innovative Research Inc.) was used for detection, and the immunosorbent signals were detected by color development, using *o*-phenylenediamine

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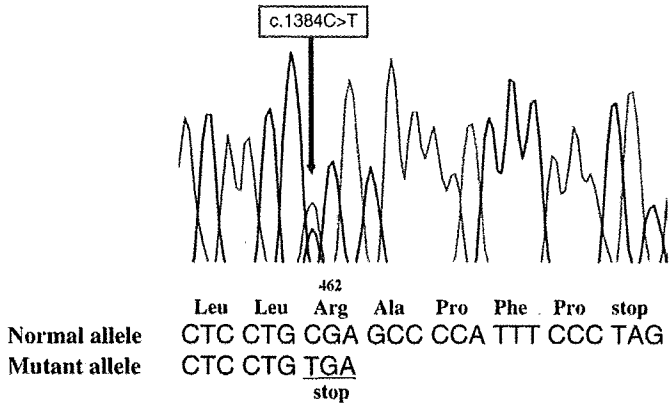


**Fig. 1.** DNA sequence analysis of the patient's FVII gene. An arrow indicates the mutation's position. A C-to-T transition at nucleotide 1384 in the coding sequence of *F7* (c.1384C>T) results in the substitution of Arg (CGA) for Term (TGA) at amino acid 462 (p.Arg462X) as a heterozygous state.

(Wako, Osaka, Japan) and 0.06% $H_2O_2$. We measured concentrations of the recombinant FVIIs in the 24 hr cultured media, and the relative amounts of FVII antigen were expressed as a percentage of levels in the wild-type sample (mean ± S.D., n = 2).

*Intracellular localization of wild-type and mutant FVIIs*

We further characterized the subcellular localization of the wild-type and mutant (Arg462X) FVIIs by immunofluorescence methods. CHO-K1 cell lines stably expressing FVIIs were seeded on glass cover slips, and fixed in 4% paraformaldehyde for 15 min. Polyclonal rabbit anti-FVII antibody, monoclonal mouse anti-Golgi-58 K-protein antibody (Sigma-Aldrich, Inc. St Louis, MO), or mouse monoclonal anti-protein disulfide isomerase (PDI) antibody (Stressgen Bioreagents, Ann Arbor, MI) was used at a dilution of 1:100 as a primary antibody. After treatment with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:400, sc-2012; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rhodamine-conjugated goat anti-mouse IgG (H + L) (1:350, Upstate Biotechnology, Lake Placid, NY), the cover slips were treated with a Slowfade antifade kit (Molecular Probes Inc, Eugene, OR) to sustain the dye's fluorescence. Cells were quickly mounted onto glass slides with Slowfade antifade reagent and subsequently analyzed with a PROVIS AX80 microscope (OLYMPUS, Tokyo, Japan).

## Results

*Case report*

The patient was a gravid Japanese woman 30 years old. She complained of vaginal bleeding episode in the 8th week of gestation and was referred to Nagoya University hospital. In laboratory tests, PT was prolonged at 17.8 sec (normal 13.4 sec), and APTT was normal at 32.6 sec (normal 32.7 sec). In other coagulation tests, only FVII:C (24%) was abnormal. She was diagnosed with a deficiency of FVII.

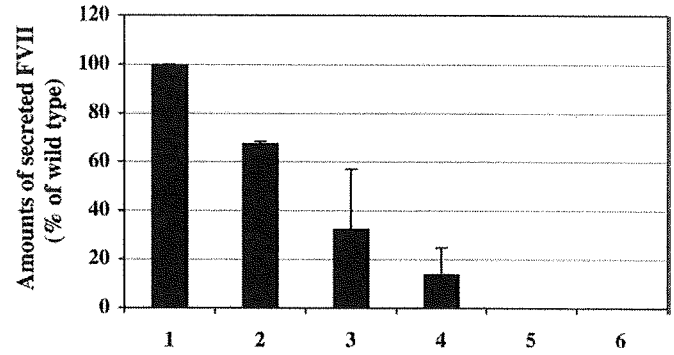


**Fig. 3.** Levels of recombinant FVIIs secreted from stably transfected CHO-K1 cells. Secretion levels of the respective recombinant FVIIs were measured by ELISA, and calculated as a percentage of those in the wild-type sample (mean ± S.D.).

*DNA analyses by direct sequencing and PCR-RFLP*

Direct sequencing of PCR fragments revealed a nonsense mutation in exon 8 of *F7* in the patient, which was a C-to-T transition at nucleotide 1384 in the coding sequence of the gene (c.1384C>T), resulting in a premature termination at 462Arg (p.Arg462X) as a heterozygous state (Fig. 1). We adopted the nomenclature recommended by Human Genome Variation Society for the description of DNA sequence variants [15], wherein the 'A' of the ATG-translation initiation codon is numbered + 1 for nucleotides, and the initial Met is numbered + 1 for amino acids. No other abnormality was found in the *F7* gene.

To confirm the mutation, a mismatch-PCR mediated *BclI*-RFLP analysis was performed as described in Materials and Methods. The PCR products treated with *BclI* showed cleaved 322 bp (mutant allele) and uncleaved 347 bp (normal allele) bands, confirming that the patient was heterozygous for this mutation (data not shown).

*Western Blot Analysis*

To clarify the role of the carboxyl-terminal region in the secretion of FVII, we performed transient expression experiments in CHO-K1 cells. A Western blot analysis of cell lysate samples of the wild-type FVII and the series of carboxyl-terminal truncated FVIIs revealed the intercellular contents of all recombinant FVII molecules to have a band of approximately 50 kDa, which corresponded to the normal size of FVII (Fig. 2(a). Western blotting of culture media of the wild-type FVII and the mutant Pro466X FVII also showed efficient secretion, but the mutants Phe465X and Pro464X were secreted inefficiently. In contrast, Ala463X and Arg462X FVIIs were not secreted into culture media at all (Fig. 2(b).

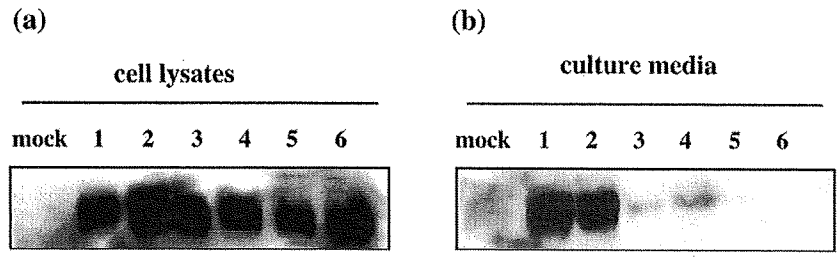


**Fig. 2.** Western blotting analysis of recombinant FVIIs. Wild-type FVII and mutant FVIIs were transiently expressed in CHO-K1 cells, and the cell lysate (a) and culture medium (b) were analyzed by Western blotting as described in Materials and Methods. Lane 1, wild-type FVII; lane 2, Pro466X FVII; lane 3, Phe465X FVII; lane 4, Pro464X FVII; lane 5, Ala463X FVII; lane 6, Arg462X FVII.

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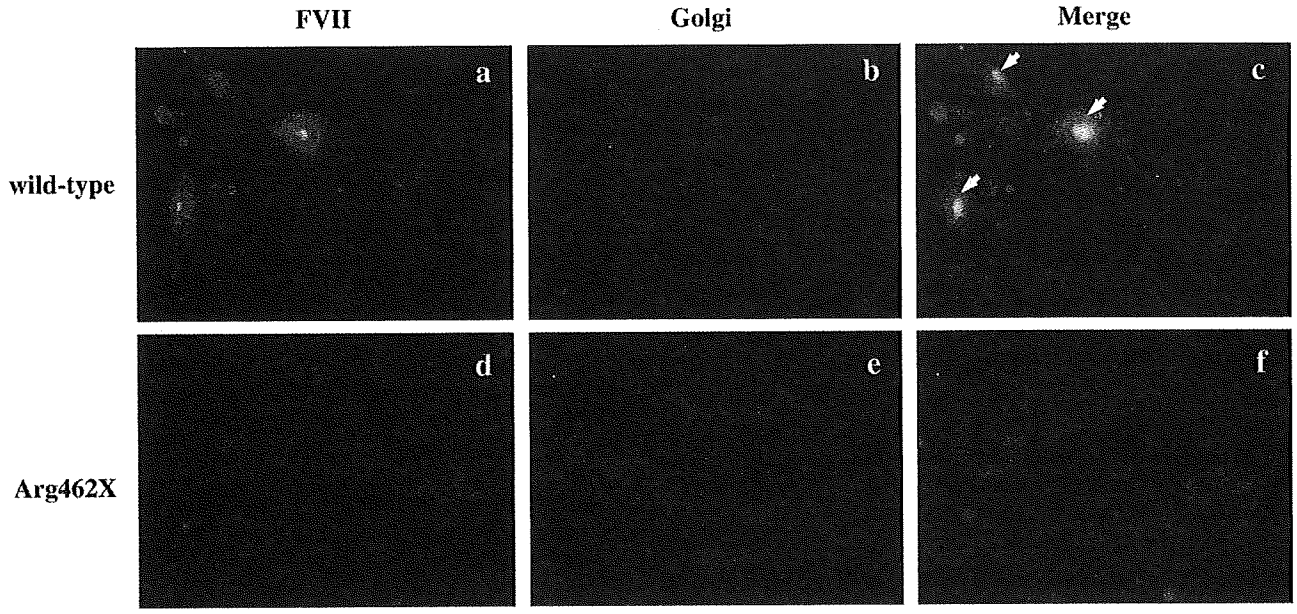


**Fig. 4.** Intracellular localization of wild-type and mutant FVIIs. The CHO-K1 cells stably expressing wild-type or mutant (Arg462X) FVII were processed for immunocytochemical staining by anti-FVII antibody (green; made visible with FITC-conjugated anti-rabbit IgG) (a, d) and anti-Golgi-58 K-protein antibody (red; visualized with rhodamine-conjugated anti-mouse IgG) (b, e). The images were merged to evaluate regions of colocalization (yellow) (c, f).

*Enzyme-linked immunosorbent assay (ELISA)*

To further study the effect of the carboxyl-terminal region on the secretion of FVII, stable transformants of CHO-K1 cells expressing the wild-type FVII or five mutants were established, and secretion levels of the recombinant FVIIs were determined by ELISA. Secretion levels were calculated as relative values, with that of the wild-type FVII taken as 100%. The secretion levels of Pro466X, Phe465X and Pro464X FVIIs were 67.7%, 32.5% and 14.1% respectively, whereas Ala463X and Arg462X FVIIs were undetectable (Fig. 3). Thus, the secretion levels of the FVII variants decreased with the length of the deletion in the carboxyl-terminal region.

*Cellular localization of wild-type FVII and FVII mutants*

To examine the intracellular localization of wild-type or Arg462X FVII in cells stably expressing them, the colocalization of the recombinant FVIIs and the Golgi apparatus was investigated using two-color confocal microscopy. In the cells expressing wild-type FVII, perinuclear spots and weak cytoplasmic reticular staining for the recombinant FVII were observed (Fig. 4a). In the cells expressing Arg462X FVII, the cytoplasmic staining for the recombinant FVII was of a similar intensity to that in the cells expressing the wild type, however, there were no perinuclear spots (Fig. 4d). The perinuclear signals for FVII were colocalized with the fluorescent signals from the Golgi in the cells expressing wild-type FVII (Fig. 4c). In contrast, most of the cytoplasmic signals for the mutant FVII did not colocalize with the signals for the Golgi in the cells expressing Arg462X FVII (Fig. 4f).

## Discussion

The molecular basis of FVII deficiency has been studied previously [6,16]. DNA abnormalities have been reported in more than 182 cases (HGMD®; http://www.hgmd.cf.ac.uk/ac/index.php), most of them caused by missense mutations, the remainder caused by nonsense mutations, frameshift mutations, or splice site abnormalities. In the present study, we identified a C-to-T transition at nucleotide 1384 in the coding sequence of *F7* (c.1384C>T), resulting in a premature termination at 462Arg (p.Arg462X), as a heterozygous state in a Japanese patient with a possible FVII deficiency. Because the p.Arg462X mutation leads to a lack of five amino acid residues in the carboxyl-terminal region of FVII, we hypothesized that it accounts for the FVII deficiency of the patient.

To test this hypothesis, we performed expression experiments in CHO-K1 cells with truncated forms of FVII based on the mutation identified in the patient. We found that the mutant Arg462X FVII was synthesized in the cells, but not secreted into the culture medium at all, in a Western blot analysis as well as ELISA. Moreover, in expression experiments with a series of truncated FVIIs, we observed that secretion levels of the variants decreased depending on the length of the carboxyl-terminal of FVII. These findings implied that the

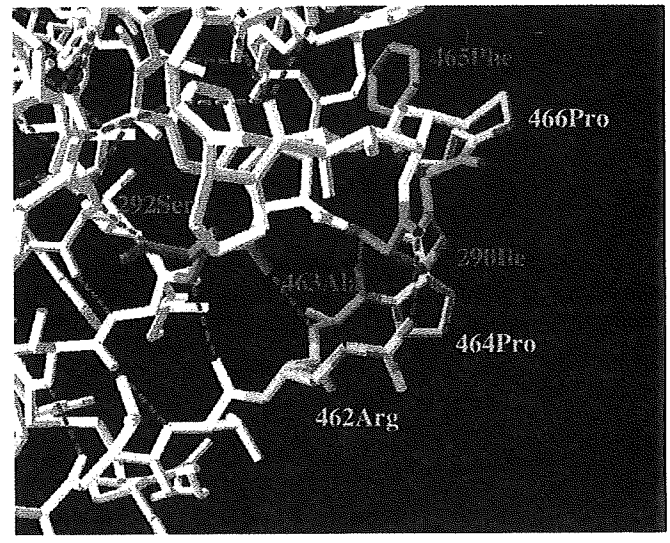


**Fig. 5.** Carboxyl-terminal structure of factor VII. The amino acids in the carboxyl-terminal region of FVII, 462Arg, 463Ala, 464Pro, 465Phe and 466Pro, are represented by a stick model in light blue, purple, light green, blue and light green, respectively. 290Ile and 292Ser are shown with red sticks. The hydrogen bonds are represented by dotted lines (yellow green). The coordinates were from the file 1AUT deposited in the Protein Data Bank. The figure was prepared using the Swiss-Pdb Viewer (http://spdbv.vital-it.ch/).

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carboxyl-terminal amino acids play important roles in the secretion of FVII.

In this study, we investigated the roles of the five amino acid residues of the carboxyl-terminal region of FVII in its secretion. A crystallographic study found that 465Phe and 463Ala would bind to 290Ile via a hydrogen bond, and 463Ala would also bind to 292Ser (Fig. 5) [17]. Therefore, the alteration of 465Phe or 463Ala to a terminal codon (Term) might cause a severe conformational change of the FVII molecule by disrupting the normal hydrogen bridging network responsible for maintaining its tertiary structure, explaining the dramatic decrease in the secretion of Phe465X FVII and Ala463X FVII. Thus, the substitution of Term for Arg at position 462 found in the patient might have a fatal effect on the secretion of FVII. Although numerous cases of hereditary FVII deficiency have provided convincing evidence that individuals with the condition tend to have an increased risk of hemorrhage, no cases had been associated with a nonsense mutation at the very end of the carboxyl-terminal, though a frameshift mutation [18] and the same mutation were reported recently [19].

Interestingly, several studies have shown the carboxyl-terminal region to be essential for the secretion and export out of the endoplasmic reticulum of certain proteins, including other vitamin K-dependent plasma glycoproteins, factor IX (FIX) and protein C (PC) [20–22]. Kurachi et al. reported that levels of intracellular and secreted FIX differed with mutations at specific amino acid residues in the carboxyl-terminal region, and that secreted levels of all mutants were lower than that of wild-type FIX. Katsumi et al. reported that extension of the carboxyl-terminal region of PC Nagoya, a variant of PC with impaired secretion due to a single nucleotide deletion in the *PROC* gene (g.8857delG) resulting in a frameshift and replacement of the carboxyl-terminal 39 normal amino acids by 81 abnormal amino acids [23], did not cause intercellular retention, but the normal carboxyl-terminal residues were required for secretion. These reports were consistent with our hypothesis that the carboxyl-terminal amino acids of FVII would play important roles in its secretion.

Intracellular protein transport is a complex process coordinated by a variety of molecules [24]. A potential mechanism to account for the reduced amounts of FVII secreted from the cells is intracellular degradation. Intracellular degradation of abnormal proteins can result from lysosomal proteolysis or from pre-Golgi or ER degradation. Our studies indicated that Arg462X FVII, which was not secreted from the cells, did not colocalize with the Golgi. The results suggested that this abnormal molecule was not transported from the ER to the Golgi, and might be retained in the ER and degraded by quality-control mechanisms for protein synthesis in the cell.

In conclusion, we investigated the molecular basis of FVII deficiency in a Japanese woman and identified a nonsense mutation (c.1384C>T: p.Arg462X) in the *F7* gene. We demonstrated that the secretion of recombinant FVIIs with carboxyl-terminal deletions, including Arg462X FVII, were impaired, suggesting the carboxyl-terminal five amino acids of FVII to be essential for its secretion, and that the c.1384C>T nonsense mutation was likely to have caused the FVII deficiency at least partly in the patient. We could not elucidate the reason for relatively low FVII level (24%) in plasma of the patient with a heterozygous p.Arg462X mutation, although it might be possible that the mutation would have a dominant-negative effect or undetectable gene variants would present on the counterpart *F7* allele. The further study is necessary to solve this issue.

## Conflict of interest statement

The authors have no conflicts of interest.

## Acknowledgments

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# Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation

Mimuro J, Mizuta K, Kawano Y, Hishikawa S, Hamano A, Kashiwakura Y, Ishiwata A, Ohmori T, Madoiwa S, Kawarasaki H, Sakata Y. Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation.
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Abstract: We studied restoration of the coagulation and fibrinolysis system in pediatric patients following liver transplantation and biomarkers of blood coagulation and fibrinolysis for suspecting the occurrence of acute cellular rejection. Coagulation activity recovered rapidly within two days following transplantation, but it took approximately 21–28 days for full recovery of the coagulation and fibrinolysis factors synthesized in the liver. PAI-1 levels were significantly higher in patients at the time of acute cellular rejection compared with levels after control of AR, and levels on days 14 and 28 in patients without AR. Plasma protein C and plasminogen levels at the time of rejection were significantly lower than those on day 14 in patients without AR. Statistical analysis suggested that an increase in plasma PAI-1 at a single time point in the post-operative period is a reliable marker among the coagulation and fibrinolysis factors for suspecting the occurrence of acute cellular rejection. These data suggested that appropriate anticoagulation may be required for 14 days after liver transplantation in order to avoid vascular complications and measurement of plasma PAI-1 levels may be useful for suspecting the occurrence of acute cellular rejection in pediatric patients following liver transplantation.

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Key words: liver transplant rejection – coagulation – fibrinolysis – plasminogen activator inhibitor 1

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Vascular thrombosis and immunological rejection of the transplanted liver in patients undergoing liver transplantation are frequent and serious post-operative complications (1). The majority of coagulation factors, factors regulating coagulation, and fibrinolysis factors are synthesized in the liver, and plasma coagulation factor levels may therefore fall for a short period after transplantation, but may return to normal levels upon regeneration of the grafted liver. Anastomosis of the vascular system of the grafted liver and the recipient vessels is carried out during liver transplantation, and anticoagulants are commonly administered for a period of time following surgery. However, restoration of the coagulation and fibrinolysis system following liver transplantation in pediatric patients has not been well studied (2, 3). In addition, the

Abbreviations: ADAMTS13, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AR, acute rejection; AST, aspartate aminotransferase; ELISA, enzyme linked immunosorbent assay; LDH, lactate dehydrogenase; NAR, no acute rejection; PAI-1, plasminogen activator inhibitor 1; PELD, pediatric model for end-stage liver disease; PT-INR, prothrombin time-international normalized ratio; sES, soluble E-selectin; TM, thrombomodulin; vWF, von Willebrand factor; γ-GTP, γ-glutamyl transpeptidase.

thrombogenic state after liver transplantation is not well understood. We performed a single center study to investigate the coagulation and fibrinolysis system and the relationship between coagulation markers and acute cellular rejection following liver transplantation from living-related donors.

## Materials and methods

### Patients and study protocol

Sixty-three pediatric patients with liver failure due to biliary atresia (n = 59), ornithine transcarbamidase deficiency (n = 2), or Wilson's disease (n = 2) underwent living-related liver transplantation from April 2001 to March 2006 and were enrolled in this study. Most of the patients with biliary atresia had previously undergone hepatic porta-jejunostomies.

### Description of patients

The patients were classified into two patient groups: one with acute cellular rejection (group AR, n = 24) and one with no acute cellular rejection (group NAR, n = 39). The diagnosis of acute cellular rejection was made by liver biopsy. There were no significant differences between group AR and group NAR in terms of age, gender, basal diseases, or the use of calcineurin inhibitors (data not shown). The PELD scores (AR, 13.0 ± 7.8; NAR, 15.1 ± 9.5), the amount of blood loss (AR, 85.0 ± 127.8 mL/kg; NAR, 125.8 ± 176.0 mL/kg), the amount of total blood transfusion (AR, 162.1 ± 109.1 mL/kg; NAR, 161.8 ± 170.8 mL/kg), the amount of plasma transfusion (AR, 64.1 ± 49.1 mL/kg; NAR, 94.2 ± 89.1 mL/kg), the cold ischemic time of graft liver (AR, 149.75 ± 126.4 min; NAR, 121.1 ± 69.1 min), and the warm ischemic time of graft liver (AR, 64.9 ± 18.1 min; NAR, 65.1 ± 13.3 min) upon operation were not significantly different between group AR and group NAR. Patients with severe infections or major bleeding episodes at the time of blood sampling for analysis were excluded from the analysis.

### Immunosuppression and anticoagulation protocols

The standard protocol for immunosuppression was as follows. Both methylprednisolone and a calcineurin inhibitor (tacrolimus or cyclosporine) were used for immunosuppression. Intravenous administration of methylprednisolone (20 mg/kg) was started during the operation and the dosage was tapered to 3 mg/kg on day 1 and to 0.5 mg/kg on day 7 after liver transplantation. A calcineurin inhibitor was infused intravenously after transplantation and the blood concentration of tacrolimus or cyclosporine was adjusted to 18–20 ng/mL or 200–300 ng/mL till day 7 after liver transplantation, respectively. Intravenous injection of calcineurin inhibitor and methylprednisolone were converted to oral administration of these regimens after patient's oral intake had been fully confirmed and the blood concentration of tacrolimus or cyclosporine was adjusted 10–15 ng/mL or 100–150 ng/mL, respectively. The methylprednisolone dose was tapered to 0.06 mg/kg on day 30. Post-operative anticoagulation was performed with intravenous administration of dalteparin (low molecular weight heparin) at the dose of 2 U/kg/h, nafamostat mesilate (serine protease inhibitor with anticoagulant activity) at the dose of 0.1 mg/kg/h, and prostaglandin E1 at the dose of 0.01 μg/kg/min till day 7 after transplantation. Anticoagulation was continued with intravenous administration of heparin (unfractionated heparin) at the dose of 8 U/kg/h from day 8 to day 21 after liver transplantation.

### Blood sample collection and analysis

All samples were obtained from patients with informed consent, according to the Declaration of Helsinki. Routine laboratory tests including complete blood counts, coagulation tests, blood chemistry analysis, and urinalysis were performed, and biomarkers of blood coagulation and fibrinolysis, i.e., PAI-1, TM, ADAMTS13, and sES were measured before and after liver transplantation on days 1, 3, 7, 10, 14, 21, and 28. Blood sampling was performed on days 35 and 49 in some patients. These were quantified using commercially available ELISAs (Mitsubishi Chemical Medience Co., Tokyo, Japan; Diaclone, Tepnel Research Products & Services, Cedex, France) (4, 5). The plasma activity levels of plasminogen and protein C were quantified using laboratory test kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Rationale for measurements of these biomarkers are as follows. PT-INR is currently used worldwide as a coagulation test to monitor the effects of anticoagulants such as coumarin in patients at risk of thrombosis. Protein C is a vitamin K-dependent protein synthesized in the liver that functions as an important regulatory factor for coagulation (6). Plasminogen is the zymogen of plasmin, a key enzyme in fibrinolysis, and is also synthesized in the liver (7). Therefore, plasma protein C and plasminogen levels were thought to be good markers for the restoration of the coagulation and fibrinolysis system following liver transplantation. Levels of these markers might correlate with protein synthesis in the liver, thereby reflecting regeneration of the graft liver. Additionally, measurement of these factors may also be important for patient management, because deficiency of protein C and type II plasminogen deficiency are thought to increase the risk of thrombosis (7, 8). The fibrin degradation product level, determined by the monoclonal antibody specific for degradation products of cross-linked fibrin, is a biomarker for the presence of a thrombus and is used to diagnose venous thrombosis and disseminated intravascular coagulation, however, the fibrin degradation product level may be affected by the presence of blood clots in the extravascular spaces (e.g., the peritoneal cavity), and may therefore not accurately reflect the thrombogenic state in the post-operative period. Thus, the soluble fibrin level was used to assess the thrombogenic state during the post-operative period following liver transplantation. PAI-1 is a primary regulator of fibrinolysis that is synthesized mainly in endothelial cells. Plasma PAI-1 levels change significantly in various pathological conditions (4). ADAMTS13 is the vWF cleaving protease that plays an important role in vWF multimer processing (9). It is synthesized in liver stellate cells and the liver is thought to be the primary source of ADAMTS13 in the circulation (9–11). In addition to the liver stellate cells, vascular endothelial cells in other organs may also be able to synthesize ADAMTS13 (12), and ADAMTS13 mRNA has been detected in the liver, kidneys and lungs in mice (13). ADAMTS13 deficiency results in platelet thrombus formation in the circulation, resulting in the development of a typical thrombotic microangiopathy (9). It is possible that ADAMTS13 deficiency might occur after liver transplantation, and plasma ADAMTS13 levels in patients were

therefore quantified following transplantation. TM, an important regulator of blood coagulation, is synthesized in vascular endothelial cells and is used as a marker of vascular injury (6). The sES level has been used as a marker for endothelial cell dysfunction (14). For example, the sES level is increased in systemic infections such as sepsis.

### Diagnosis of acute cellular rejection

The diagnosis of acute cellular rejection was made by liver biopsy and was evaluated using the rejection activity index (3) scores (1, 15, 16). Patients suspected of suffering from acute cellular rejection because of deterioration of liver function (increased serum levels of bilirubin, AST, ALT, ALP, LDH, and γ-GTP compared with previous levels) were subjected to ultrasonography-guided liver biopsy. The liver biopsy specimens were examined for the presence of acute cellular rejection. Patients diagnosed with acute cellular rejection were subjected to intensive immunosuppressive therapy with intravenous methylprednisolone. Mycophenolate mofetil and/or OKT3 were also administered in some patients. Plasma samples obtained before starting administration of the intensive immunosuppressive regimens were evaluated in the following studies.

### Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Tokyo, Japan). Student *t*-tests were used to compare the mean values between groups. Multiple logistic regression analysis was used to investigate the association between biomarkers of blood coagulation and fibrinolysis and the occurrence of acute cellular rejection. p-values $< 0.05$ were considered statistically significant.

## Results

### Analysis of the coagulation and fibrinolysis system following liver transplantation

Changes in mean values of coagulation tests in patients without acute cellular rejection, vascular complications, or severe infections are shown in Fig. 1. The coagulation activity after liver transplantation was assessed by measuring prothrombin time (PT-INR). The mean PT-INR value rose to approximately 1.8 on day 1, but quickly fell again to $< 1.5$ on day 2, and then normalized gradually. These data suggest that the coagulation activity rapidly recovered after transplantation, once the graft liver started to function.

The mean protein C level of patients before liver transplantation decreased to 57.5% of the normal level. This may have been due to the decreased synthesis of protein C in the liver because most patients had liver failure. The mean protein C level fell to approximately 50% of the normal level on day 1 post-transplantation, and then increased gradually, reaching ≥80% of the normal level by day 14. The mean plasminogen level changed in a similar manner to protein C. By day 28, both protein C and plasminogen levels had returned to almost 90–100% of the normal levels. The nadir values of protein C and plasminogen on day 1 post-transplantation might

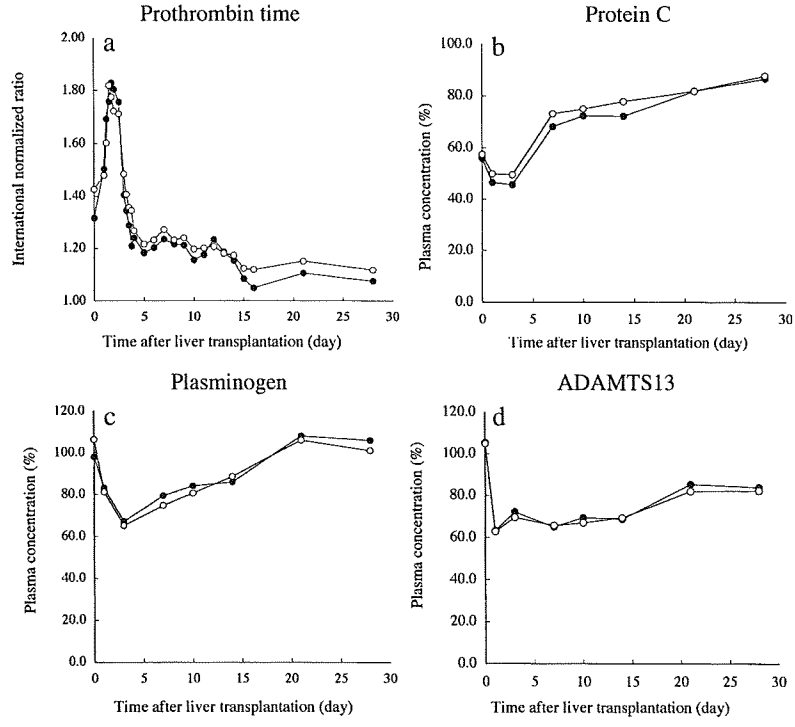


*Fig. 1.* Restoration of the coagulation and fibrinolysis system following liver transplantation. (a) The mean values of coagulation activity assessed by PT-INR and (b) plasma levels of protein C (normal range 67.1–129.0%), (c) plasminogen (normal range 85.0–120.0%), and (d) ADAMTS13 (normal range 100 ± 15%) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

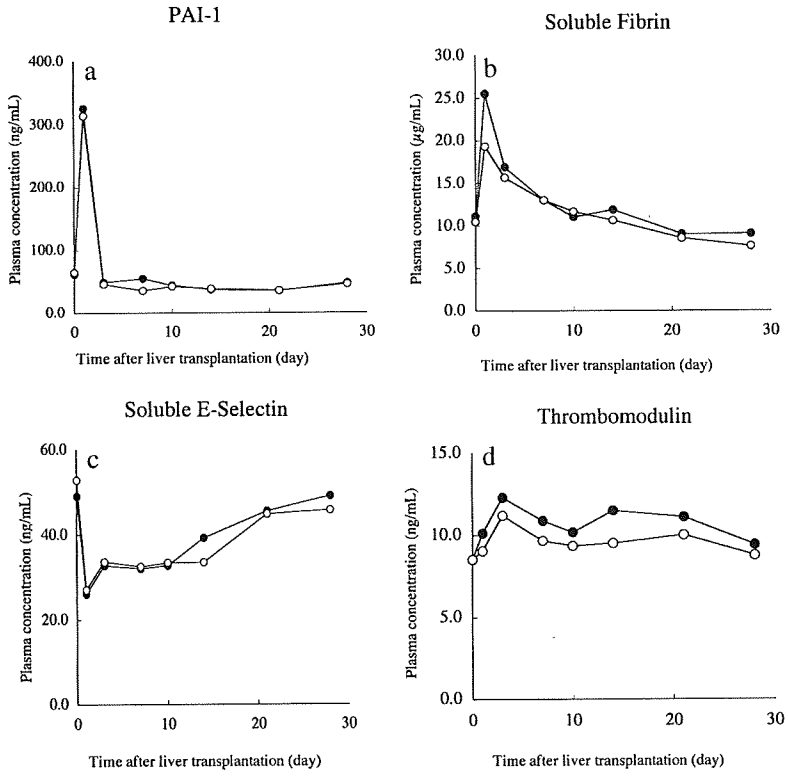


*Fig. 2.* Analysis of biomarkers of coagulation and fibrinolysis following liver transplantation. The mean plasma levels of PAI-1 (normal range 20–30 ng/mL), soluble fibrin (normal range: < 7.5 μg/mL), sES (normal range: < 37.5 ng/mL), and TM (normal range 4.46 ± 1.36 ng/mL) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

be affected by plasma transfusion during and after surgery. These data suggest that the synthesis of coagulation factors in the graft liver may start on day 1, resulting in rapid recovery of coagulation activity, but it may take up to 14 days for recovery of the coagulation and fibrinolysis system to near normal levels, and 21–28 days for full restoration of the system after liver transplantation. These data also suggest that graft livers may regenerate to the appropriate size within four wk, though graft livers may vary in size depending on their recipients and donors. The average levels of the coagulation and fibrinolysis factors in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods, but rate of restoration of the protein C and plasminogen levels on day 14 in group AR was slow.

Soluble fibrin levels in patients with no complications increased significantly on day 1 and then gradually decreased, normalizing by day 14 as shown in Fig. 2. These data suggest that the thrombotic state may continue for 14 days after liver transplantation, and that appropriate antithrombotic therapy may therefore be required during this period.

The mean plasma PAI-1 level was increased approximately 10-fold on post-operative day 1, compared with the normal level, but returned quickly to the normal level on day 3 after transplantation (Fig. 2). These data, together with the changes in the plasminogen level during the post-operative period (Fig. 1), suggest that fibrinolysis activity was suppressed on day 1 after liver transplantation.

The average plasma ADAMTS13 level decreased significantly on day 1 post-transplantation (Fig. 2), but the decrease was not as severe as that of protein C or plasminogen (Fig. 1). However, low levels of ADAMTS13 were maintained for 14 days after liver transplantation. These changes in plasma ADAMTS13 levels after liver transplantation did not parallel those of protein C or plasminogen (Fig. 1), reflecting the extrahepatic synthesis of ADAMTS13 and the possibility that ADAMS13 is synthesized not in hepatocytes, but in stellate cells in the liver. The plasma ADAMTS13 level fell to 28.4% of the normal level in one patient, but she showed no typical signs of thrombotic microangiopathy.

The TM level was increased on day 3 post-transplantation and remained at the upper limit of the normal range after day 7 (Fig. 2). The sES level was significantly increased in patients before liver transplantation (Fig. 2), which may be explained by the fact that many patients enrolled

in the study had undergone hepatic porta-jejunostomies and therefore had biliary tract infections before transplantation. The sES level was reduced post-transplantation, and remained almost within the normal range until day 14, but then was significantly increased on days 21 and 28 (Fig. 2). This increase in the sES level was not associated with the presence of infection or other disease states. The average changes of biomarkers of the coagulation and fibrinolysis system in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods.

Three patients in this study suffered from hepatic artery thrombosis after liver transplantation, and an increased PT-INR (prolongation of prothrombin time) was detected in all three patients. Increase of plasma PAI-1 in the following samples of two patients was observed. Decrease of plasma protein C and plasma ADAMTS 13 in the following samples were observed in two patients. Other biomarkers did not change significantly. However, due to the small sample size, the predictive value of this test for the development of vascular complication was inconclusive.

### Relationship between coagulation and fibrinolysis markers and acute cellular rejection

Patients were divided into two groups, group AR and group NAR, based upon the presence of acute cellular rejection as described above. The mean onset time of acute cellular rejection in group AR was on day 15 ± 8.7 after liver transplantation, while the mean time for data collection was on day 14 ± 7.9. Laboratory data and coagulation markers for each group at two time points were subjected to statistical analysis. Measurements taken immediately before the diagnosis of acute cellular rejection in group AR were compared with those taken after the cessation of rejection by intensive treatment with methylprednisolone in group AR, and those taken on days 14 and 28 in group NAR.

Statistical analysis of the mean levels of coagulation and fibrinolysis markers (Table 1) revealed that the PAI-1 level at the time of acute cellular rejection in group AR was significantly higher than that after cessation of rejection in group AR, and those on days 14 and 28 in group NAR (data for day 28 of group NR are not shown in Table 1).

The plasma protein C and plasminogen levels at the time of AR diagnosis in group AR were significantly lower than those on day 14 in group NAR.

Table 1. Coagulation and fibrinolysis biomarkers following liver transplantation

| | Group AR (n = 24) | | Group NAR (n = 39) |
|---|---|---|---|
| | Before* | After† | Day 14 |
| PAI-1 (ng/mL) | 79.3 ± 103.9‡ | 23.0 ± 10.7 | 38.5 ± 30.4 |
| Plasminogen (%) | 85.2 ± 22.8‡ | 99.4 ± 29.0 | 97.68 ± 13.8 |
| Protein C (%) | 65.7 ± 23.0‡ | 89.3 ± 37.9 | 87.2 ± 25.5 |
| ADAMTS13 (%) | 67.5 ± 24.1 | 77.8 ± 23.6 | 72.5 ± 17.4 |
| ATIII (%) | 96.3 ± 17.3 | 111.5 ± 57.4 | 99.3 ± 14.9 |
| PT-INR | 1.17 ± 0.21 | 1.08 ± 0.13 | 1.13 ± 0.13 |
| Fibrinogen (mg/mL) | 295.3 ± 116.4 | 296.3 ± 106.7 | 280.6 ± 74.0 |
| Thrombomodulin (U/mL) | 10.2 ± 3.8 | 10.8 ± 4.8 | 8.7 ± 5.2 |
| Soluble E-selectin (μg/mL) | 43.8 ± 16.7 | 46.4 ± 19.0 | 33.5 ± 17.2 |
| Soluble fibrin (μg/mL) | 13.57 ± 17.3 | 8.64 ± 14.9 | 10.2 ± 13.9 |

*Values at the time immediately before acute cellular rejection.
†Values after cessation of acute cellular rejection.
‡Values taken from the time point proximate to acute cellular rejection (before) are significantly different from those of group AR after cessation of acute cellular rejection (after) and those on day 14 in group NAR ($p < 0.01$).
Values are mean ± s.d.

The ADAMTS13 level at the diagnosis of AR in group AR appeared to be lower than that after cessation of rejection in group AR, and those on day 14 in group NR, though the differences were not statistically significant.

There were no significant differences between the levels of other coagulation and fibrinolysis markers in patients at the time of rejection diagnosis and after cessation of acute cellular rejection in group AR, or the levels on days 14 and 28 in group NAR.

The changes of the coagulation and fibrinolysis factors and biomarkers before the diagnosis of acute cellular rejection by liver biopsy were studied. These biomarkers levels of samples obtained from the patients proximate to the diagnosis of acute cellular rejection (AR-proximate sample in Fig. 3) were compared with those obtained before the AR-proximate sample (earlier sample in Fig. 3). The PAI-1 level in the AR-proximate samples were significantly higher than that in the earlier samples. The mean values of protein C, plasminogen, and ADAMTS13 in the AR-proximate samples was expected to be higher than those in the earlier samples, but they were lower than the earlier samples though the differences were not statistically significant. The mean values of other biomarkers in the two time points were not significantly different.

Multiple logistic regression analysis was performed to identify the coagulation and fibrinolysis markers for suspecting the occurrence of acute cellular rejection. Absolute values of coagulation and fibrinolysis factors (protein C, plasminogen, ADAMTS13) synthesized in the liver

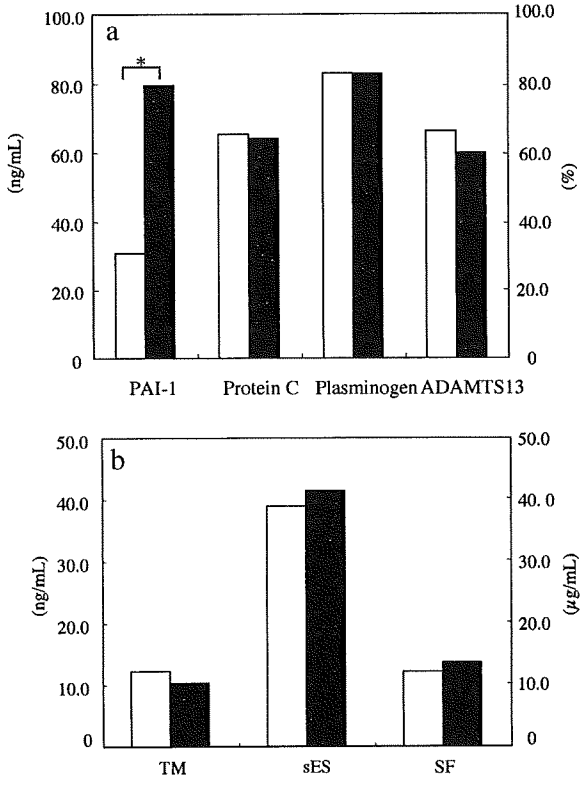


*Fig. 3.* Changes of coagulation and fibrinolysis factors and biomarkers in patients with acute cellular rejection. The mean plasma levels of coagulation and fibrinolysis factors and biomarkers obtained from patients with acute cellular rejection at two time points were shown. The AR-proximate samples (closed square) were obtained from the patients proximate to the diagnosis of acute cellular rejection. The earlier samples (open square) were obtained before the AR-proximate samples.

were difficult to ascertain using this method, and the changes in levels between time points were therefore analyzed. PAI-1 levels are independent of regeneration of the liver and an increase in plasma PAI-1 levels of >40 ng/mL at a single time point was therefore taken into account. Increases in soluble fibrin, TM, or sES since the previous time point, and above the normal range, were also taken into account. A summary of the multiple logistic regression analysis of coagulation and fibrinolysis markers is shown in Table 2. These data suggest that an increase in PAI-1 levels, and a decrease in protein C, plasminogen, or ADAMTS13 levels, were independently related to the occurrence of acute cellular rejection. Other markers were not related to the occurrence of acute cellular rejection (Table 2). Among these markers, an increase in plasma PAI-1 levels was observed in almost 80% of the patients in group AR.

Table 2. Multiple logistic regression analysis of biomarkers

| | Odds ratio | Confidence interval | p-value |
|---|---|---|---|
| PAI-1 | 17.91 | 4.89–64.36 | <0.001 |
| ADAMTS13* | 6.40 | 1.85–22.03 | 0.003 |
| Protein C* | 4.58 | 1.14–18.28 | 0.027 |
| Plasminogen* | 7.86 | 1.23–49.83 | 0.02 |
| Soluble fibrin† | 0.60 | Not applicable | 0.60 |
| Soluble E-selectin† | 0.60 | Not applicable | 0.65 |
| Thrombomodulin† | 0.421 | Not applicable | 0.42 |

*Decrease of marker values from the previous time point was adopted.
†Increase of marker values from the previous time point was adopted.

## Discussion

The prevention and treatment of vascular thrombosis and immunological rejection of the transplanted liver during the post-operative period is a keystone of patient management. The present study analyzed the coagulation and fibrinolysis system following liver transplantation in pediatric patients to identify biomarkers for suspecting the occurrence of acute cellular rejection.

The present study suggests that the coagulation activity recovered rapidly once the graft liver started functioning, and that the graft liver might regenerate to the appropriate size in 21–28 days, with coincident full recovery of the coagulation and fibrinolysis system in pediatric patients undergoing liver transplantation. The present study also suggests that the hypercoagulable state persisted for 14 days after surgery, and that appropriate anticoagulation may therefore be required at least for 14 days post-transplant, even in the absence of any apparent vascular complications.

Recent advances in the management of patients with liver transplants have improved the clinical outcome of these patients. Adjustments in the doses of immunosuppressive drugs such as calcineurin inhibitors, based on their blood concentrations, are widely conducted after liver transplantation. However, immunological rejection of the transplanted liver still develops in a certain ratio of these patients, even when the blood calcineurin inhibitor concentration is within the appropriate therapeutic range (17, 18). A variety of methods for evaluating immune cell activation have been proposed as a basis for adjusting immunosuppressive therapy, and these have been shown to be useful for assessing the level of immunosuppression (19–22). Intensive treatment of acute cellular rejection with high dose methylprednisolone, with or without other medicines such as OKT3, is usually effective, though the prediction and rapid diagnosis of AR may be important for its effective treatment. In