

# A case of thrombomodulin mutation causing defective thrombin binding with absence of protein C and TAFI activation

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## Key Points

- We herein report the first case of functional TM deficiency resulting from a mutation in the thrombin-binding domain of the TM gene.

Thrombomodulin functions as an anticoagulant through thrombin binding and protein C activation. We herein report the first case of hereditary functional thrombomodulin deficiency presenting with recurrent subcutaneous hemorrhage and old cerebral infarction. The patient had a homozygous substitution of glycine by aspartate at amino acid residue 412 (Gly412Asp) in the thrombin-binding domain of the thrombomodulin gene (designated thrombomodulin-Nagasaki). In vitro assays using a recombinant thrombomodulin with the same mutation as the patient showed a total lack of thrombin binding and activation of protein C and thrombin-activatable fibrinolysis inhibitor (TAFI). Marked clinical and laboratory improvement was obtained with recombinant human soluble thrombomodulin therapy.

## Introduction

Thrombomodulin (TM), a thrombin-binding protein on the surface of vascular endothelial cells, functions as a modulator of coagulation and inflammation.<sup>1-3</sup> TM enhances thrombin-catalyzed activation of protein C, and activated protein C (APC) proteolytically inactivates blood coagulation factors Va and VIIIa.<sup>4</sup> TM also enhances the thrombin-catalyzed activation of thrombin-activatable fibrinolysis inhibitor (TAFI), and activated TAFI inactivates complement factor C5, which inhibits inflammation.<sup>5</sup> Recombinant human soluble TM (Recomodulin), which is composed of an extracellular region of TM and possesses the same activities as native TM,<sup>6</sup> has been used to treat disseminated intravascular coagulation in Japan.<sup>7-9</sup>

Single nucleotide polymorphisms of the TM gene have been associated with thrombophilic tendency and complement-mediated thrombotic microangiopathy.<sup>10,11</sup> Animal studies have shown that mice with mutations in the thrombin-binding domain of TM have an extremely reduced ability to produce APC,<sup>12,13</sup> indicating that mutations in this domain may be detrimental for its role as an anticoagulant. However, there are no reports of similar mutations of the human TM gene. We report the first case of functional TM deficiency resulting from a homozygous substitution mutation in the thrombin-binding domain of the TM gene (designated TM-Nagasaki). It may present a new entity of thrombophilia syndrome.

## Methods

### Antibodies and 2 TM ELISA systems

Two enzyme-linked immunosorbent assays (ELISA) were used to determine the plasma TM concentration. One was composed of polyclonal antihuman TM-rabbit F(ab') (used as the solid-phase antibody) and antihuman TM-rabbit F(ab') coupled with  $\beta$ -D-galactosidase (used as the second antibody); it was designated as polyclonal antibody ELISA (pAb-ELISA).<sup>6</sup> The other was composed of

MFTM-4, a monoclonal antihuman TM antibody recognizing the thrombin-binding site<sup>14</sup> (used as the solid-phase antibody) and antihuman TM-rabbit F(ab') coupled with  $\beta$ -D-galactosidase (used as the second antibody); it was designated as monoclonal antibody ELISA (mAb-ELISA).<sup>15</sup> Recomodulin, provided by Asahi Kasei Pharma Corporation (Tokyo, Japan), was used as a standard substance in mAb-ELISA and pAb-ELISA. While mAb-ELISA measures concentrations of TM degradation products containing the thrombin-binding site (TBS) region of the TM molecule exclusively, pAb-ELISA measures those containing not only the TBS region but also other regions of the TM molecule. Accordingly, reference values of healthy controls in the former (2.3 to 3.7 ng/mL) are lower than those in the latter (12.1 to 24.9 ng/mL).

### Preparation of cells, RNA, and DNA

This study was approved by the Nagasaki University Hospital ethics committee (approval number: 11092631). Written informed consent was obtained from the patient and his family in agreement with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) were isolated from the patient, his family members, and a healthy control. Total RNA and genomic DNA were extracted from PBMCs with a QIAamp RNA kit and a QIAamp blood kit (Qiagen), respectively.

### Analysis of TM mRNAs and TM gene sequencing

Reverse transcription was performed with random hexamer primers (Takara Shuzo, Kyoto, Japan). PCR amplification of the TM gene was performed with sense (5'-AAGTGAAGGCCGATGGCTTC-3') and antisense (5'-TTGGGAACGCAGAAGTGCTC-3') primers designed by the authors (M.O.). The full-length TM gene was amplified by PCR using a set of primers, as described previously.<sup>16</sup> PCR products were sequenced with an ABI Prism Dye Terminator sequencing kit (Perkin-Elmer/Applied Biosystems), according to the manufacturer's instructions.

### Preparation of recombinant TMs

DNA fragments coding wild-type human soluble TM composed of an extracellular region of TM (residues Ala1 to Ser497) and human soluble TM with substitution of glycine by aspartic acid at amino acid residue 412 (Gly412Asp) were synthesized and cloned into the pPICZ $\alpha$ A expression vector (Invitrogen). These vectors were transfected into *Pichia pastoris* strain SMD1163 to express wild-type recombinant human soluble TM (rhsTM) and rhsTM with Gly412Asp mutation (rhsTM G412D). The generated recombinant proteins were purified with Ni Sepharose 6 Fast Flow (GE Healthcare).<sup>17</sup>

### Polyacrylamide gel electrophoresis and western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 4% to 15% gradient gel (Bio-Rad, Tokyo, Japan) under reduced conditions.<sup>6</sup> Proteins were stained with Bullet CBB Stain One (Nacalai Tesque, Kyoto, Japan). Western blotting was performed as described.<sup>6,14</sup> After SDS-PAGE, recombinant TMs transferred onto a nitrocellulose membrane were detected by treatment first with biotinylated anti-TM rabbit antibody and next with streptavidin alkaline phosphatase (Promega, Madison, WI) and then with western blue stabilized substrate for alkaline phosphatase (Promega).

### Measurement of clotting times

The effects of rhsTM or rhsTM G412D on the prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin clotting time (TCT) were measured using a blood coagulation time measuring device (CA-50; SYSMEX, Kobe, Japan) according to the manufacturer's instructions. For the PT assay, the clotting time was measured after the addition of solution containing various concentrations of rhsTM or rhsTM G412D (5  $\mu$ L), normal human plasma (45  $\mu$ L), and Thromborel S (Siemens) containing tissue factor and CaCl<sub>2</sub> (100  $\mu$ L). For the APTT assay, the clotting time was measured after incubating the mixture consisting of solution containing various concentrations of rhsTM or rhsTM G412D (5  $\mu$ L), normal human plasma (45  $\mu$ L), and actin (50  $\mu$ L; Siemens) for 2 minutes. Then 2 mM CaCl<sub>2</sub> (50  $\mu$ L) was added to the reaction mixture. For the TCT assay, the clotting time was measured after incubating 100  $\mu$ L of reaction mixture consisting of normal human plasma (10  $\mu$ L), Owren veronal buffer (80  $\mu$ L), and solution containing various concentrations of rhsTM or rhsTM G412D (10  $\mu$ L) for 3 minutes. Then 50  $\mu$ L of 5  $\mu$ g/mL thrombin (Mochida Pharmacy, Tokyo, Japan) was added.

### TGT

The effect of rhsTM or rhsTM G412D on thrombin generation was determined by thrombin generation test (TGT) using a calibrated automated thrombogram system (Thrombinoscope BV, Maastricht, Netherlands)<sup>18</sup> according to the manufacturer's instructions. Briefly, 20  $\mu$ L of PPP-reagent (Thermo-Fisher Scientific, Waltham, MA) containing 15 pM tissue factor and 24  $\mu$ M phospholipid vesicles was added to the mixture of normal human plasma (72  $\mu$ L) and various concentrations of rhsTM or rhsTM G412D (8  $\mu$ L) and FluCa-Kit (Thermo-Fisher Scientific; 20  $\mu$ L) containing 2.5 mM thrombin substrate (z-Gly-Gly-Arg-7-amino-4-methylcoumarin) and 100 mM CaCl<sub>2</sub>. Generated thrombin was monitored in real time as the amount of fluorescent aminomethyl coumarin.

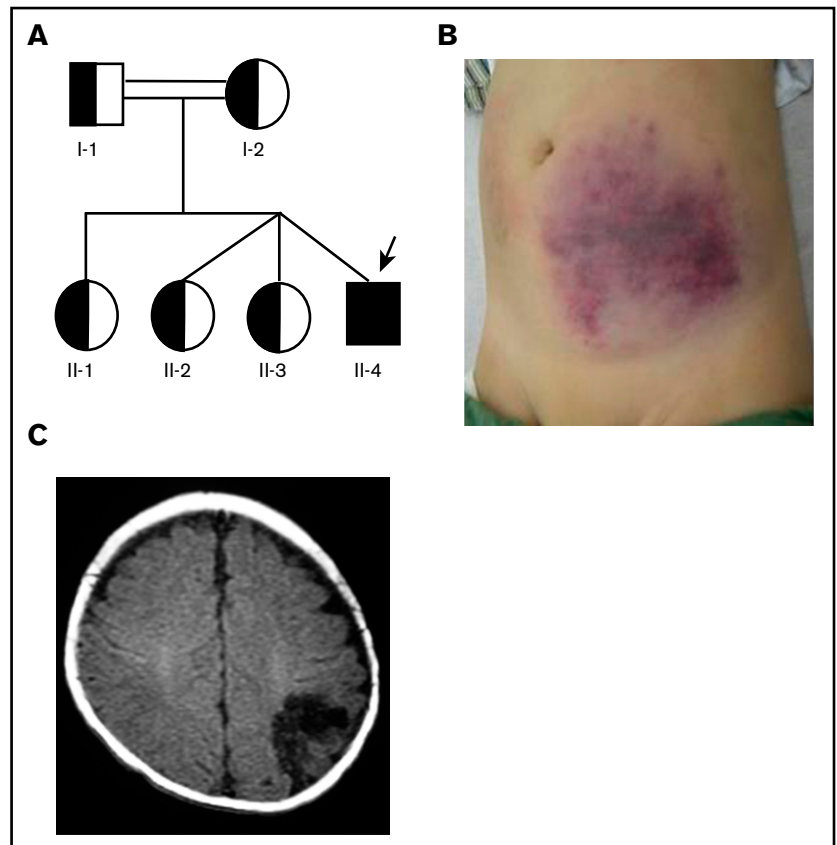
### Measurement of the protein C-activating cofactor activity of recombinant TMs

The effects of the cofactor activity of rhsTM or rhsTM G412D on thrombin-catalyzed protein C activation were determined as described.<sup>6</sup> Briefly, a 100- $\mu$ L reaction mixture containing thrombin (0.25  $\mu$ g/mL), protein C (25  $\mu$ g/mL), and various concentrations of Recomodulin, rhsTM, or rhsTM G412D was incubated for 60 minutes. The reaction was stopped by adding 50  $\mu$ L of 100  $\mu$ M argatroban, a thrombin-specific inhibitor (Tokyo Chemical Industry, Tokyo, Japan). Then the APC activity was determined using a specific substrate, Glu-Pro-Arg-pNA (S-2366; Chromogenix). The p-nitroanilide (pNA) liberated by APC for 15 minutes was determined at 405 nm with a microplate reader (Ultrospec 2100pro; Amersham Biosciences).

### Measurement of the TAFI-activating cofactor activity of recombinant TMs

The effect of rhsTM or rhsTM G412D on the thrombin-catalyzed activation of TAFI was determined by the previously described method.<sup>19</sup> In brief, 100  $\mu$ L of reaction mixture containing thrombin (0.05  $\mu$ g/mL), TAFI (4.5  $\mu$ g/mL; Haematologic Technologies, Essex Junction, VT), and various concentrations of Recomodulin, rhsTM, or rhsTM G412D (0-10  $\mu$ g/mL) was incubated at 37°C for 30 minutes. The reaction was stopped by adding 5  $\mu$ L of 500  $\mu$ M

**Figure 1. The patient's signs and family pedigree.** (A) The patient's family pedigree. The patient is a fraternal triplet and his parents were second cousins. He is homozygous for G1289A mutation of the TM gene. Other family members are heterozygous for this mutation. (B) Subcutaneous bleeding that repeatedly appeared almost every week. (C) Brain computed tomography scan showing an old cerebral infarction, possibly due to embolism with a blood clot that was formed in the venous system and shifted to an arterial current through the foramen ovale during the fetal period.



argatroban, and then 10  $\mu\text{L}$  of the reaction mixture was mixed with 5  $\mu\text{L}$  of hippuryl-L-Arg (30 mM) and incubated at 37°C for 60 minutes. The reaction was stopped by adding 75  $\mu\text{L}$  of 0.25 M phosphate buffer (pH 8.3) and 100  $\mu\text{L}$  of cyanuric chloride in 1,4-dioxane, and then the mixture was centrifuged at 20 000g for 10 minutes at room temperature. The supernatant (100  $\mu\text{L}$ ) was transferred to a 96-well microplate, and the absorbance at 405 nm was measured using a microplate reader (iMark Microplate Reader; Bio-Rad).

### Measurement of thrombin binding ability to recombinant TMs

The binding of thrombin to rhsTM or rhsTM G412D was determined as described previously.<sup>20</sup> Briefly, rhsTM or rhsTM G412D (10  $\mu\text{g}/\text{mL}$ ) was fixed in the well of a 96-well microplate, and 100  $\mu\text{L}$  of thrombin (0-5  $\mu\text{g}/\text{mL}$ ) was added to the wells and incubated for 1 hour. After washing the wells, 100  $\mu\text{L}$  of 1 mM H-D-Phe-Pip-Arg-pNA (S-2238), a thrombin-specific substrate (Sekisui Medical, Tokyo, Japan) was added and incubated for 10 minutes. The pNA liberated by thrombin was determined as described above.

### Statistical analyses

Data were collected from 3 independent experiments and expressed as means  $\pm$  standard deviation. Statistical analyses were performed using an analysis of variance followed by a Student *t* test to identify significant differences in multiple comparisons. Differences were considered statistically significant at  $P < .01$ .

## Results

### Case Presentation

The patient (Figure 1A, II-4), a 6-month-old boy, was referred to our hospital because of recurrent subcutaneous bleeding (Figure 1B). His parents (Figure 1A, I-1 and I-2) were consanguineous (second cousins), but his family history revealed no bleeding tendency or thrombophilia within third degrees of kinship to the patient. Neither his family members nor relatives had a history of chronic inflammatory disease. His mother did not report a history of spontaneous abortion. He had no significant perinatal history even though he was a triplet. At 3 months of age, he started experiencing subcutaneous bleeding, which mainly appeared on the trunk and which spontaneously disappeared after several days.

The laboratory tests of the patient in a usual state (Table 1; baseline at 3 months of age) showed marked coagulation-fibrinolysis system abnormalities: fibrinogen/fibrin degradation products (FDP), 77.7  $\mu\text{g}/\text{mL}$  (normal  $<5$ ); D-dimer, 32.9  $\mu\text{g}/\text{mL}$  ( $<1.0$ ); thrombin-antithrombin complex (TAT),  $>60$  ng/mL ( $<3.0$ ); and plasmin- $\alpha$ 2plasmin complex (PIC), 10.2 ng/mL ( $<0.8$ ). His platelet counts ( $236 \times 10^3/\mu\text{L}$ ) and bleeding time (2.0 minutes) were normal, and his protein C activity was also normal for his age. Additional tests also revealed a hypercoagulation state in which the fibrin monomer complex concentration was  $>250$   $\mu\text{g}/\text{mL}$  (normal,  $<6.1$   $\mu\text{g}/\text{mL}$ ) and prothrombin fragments (F1+F2) were 26.6 nmol/L (normal range, 0.44 to 1.20 nmol/L). The concentration of soluble TM, which was measured by mAb-ELISA during the screening of vascular endothelial dysfunction, was  $<1.0$  ng/mL (below the detection

**Table 1. Laboratory data of the patient**

	Normal ranges	Baseline (age, 3 mo)	Baseline (age, 10 y)	During a bleeding episode	2 d after rTM (380 U/kg)
PLT, $\times 10^3/\mu\text{L}$	130-320	236	207	124	292
PT-INR	0.85-1.15	0.89	1.45	1.61	1.13
APTT, s	24.3-36.0	29.2	43.2	42.2	32.8
Fibrinogen, mg/dL	150-400	57	51	36	49
FDP, $\mu\text{g/mL}$	<5	77.7	65.7	198.2	6.3
D-dimer, $\mu\text{g/mL}$	<1.0	32.9	20.6	60.8	3.2
Protein C activity, %	46-156	41	96	106	104
TAT, ng/mL	<3.0	>60.0	>60.0	>60.0	6.2
PIC, $\mu\text{g/mL}$	<0.8	10.2	10.2	13.4	1.1
TM, ng/mL*	2.3-3.7	<1.0	<1.0	<1.0	>201

PLT, platelet count; PT-INR: prothrombin test–international normalized ratio.

\*The plasma TM concentration was measured by used a monoclonal antihuman TM antibody recognizing the thrombin-binding site.<sup>14</sup>

limit). Purpuric skin lesions (diameter, 50 to 100 mm) repeatedly appeared over the trunk and extremities almost every week (Figure 1B) and disappeared after a few days without any medical intervention in most cases. No consumptive coagulopathy, such as organ failure or asymptomatic thrombosis, had appeared for years except for an old cerebral infarction (Figure 1C) recognized in the infantile period that might have been formed by a venous thrombus through the foramen ovale, which is open during the prenatal stage. Occasionally, hemostasis was difficult to achieve after minor injuries (Table 1; during a bleeding episode). At such times, the administration of Recomodulin (380 U/kg, in a single dose) markedly improved his clinical symptoms and abnormal laboratory data (Table 1; 2 days after rTM). At the time of his last visit, the patient was 12 years of age and his growth and development had been normal without any apparent episodes of thrombosis.

### Expression of TM mRNA from the TM gene

RT-PCR showed the comparable expression of TM mRNA in PBMCs obtained from the patient and a healthy control (Figure 2A).

### The plasma concentration of soluble TM protein

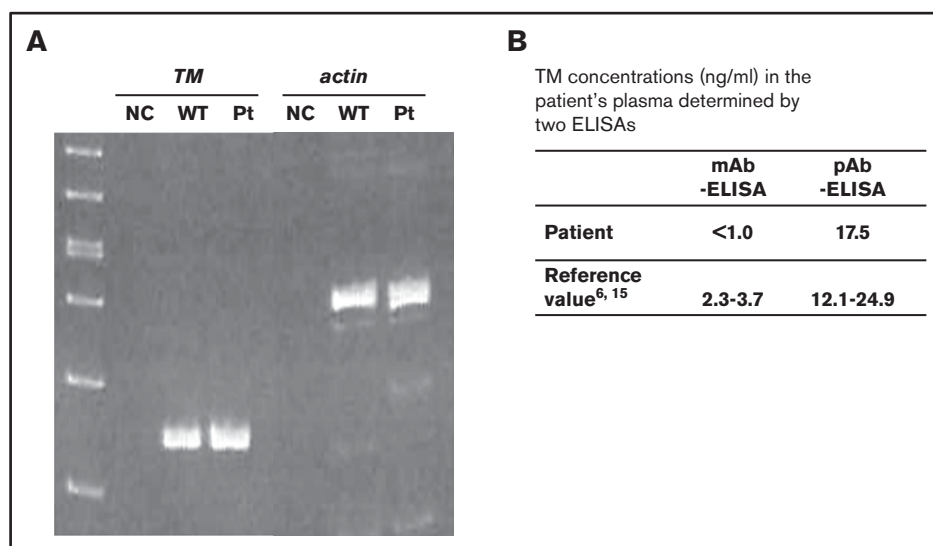
Soluble TM was not detected in the patient's plasma (<1.0 ng/mL) by mAb-ELISA; however, a healthy control level (17.5 ng/mL) was detected by pAb-ELISA (Figure 2B).

### TM gene sequences

The abovementioned contradictory data on TM protein concentrations determined by the 2 ELISAs suggested that the patient's TM gene had a mutation or multiple mutations at the thrombin-binding domain. Direct sequencing of the patient's TM gene revealed a homozygous G1289A mutation (Figure 3F), resulting in the substitution of Asp for Gly at residue 412 (G412D) in the thrombin-binding epidermal growth factor (EGF)5 domain. Other family members (parents and 3 sisters) were heterozygous for this mutation (Figure 3A-E).

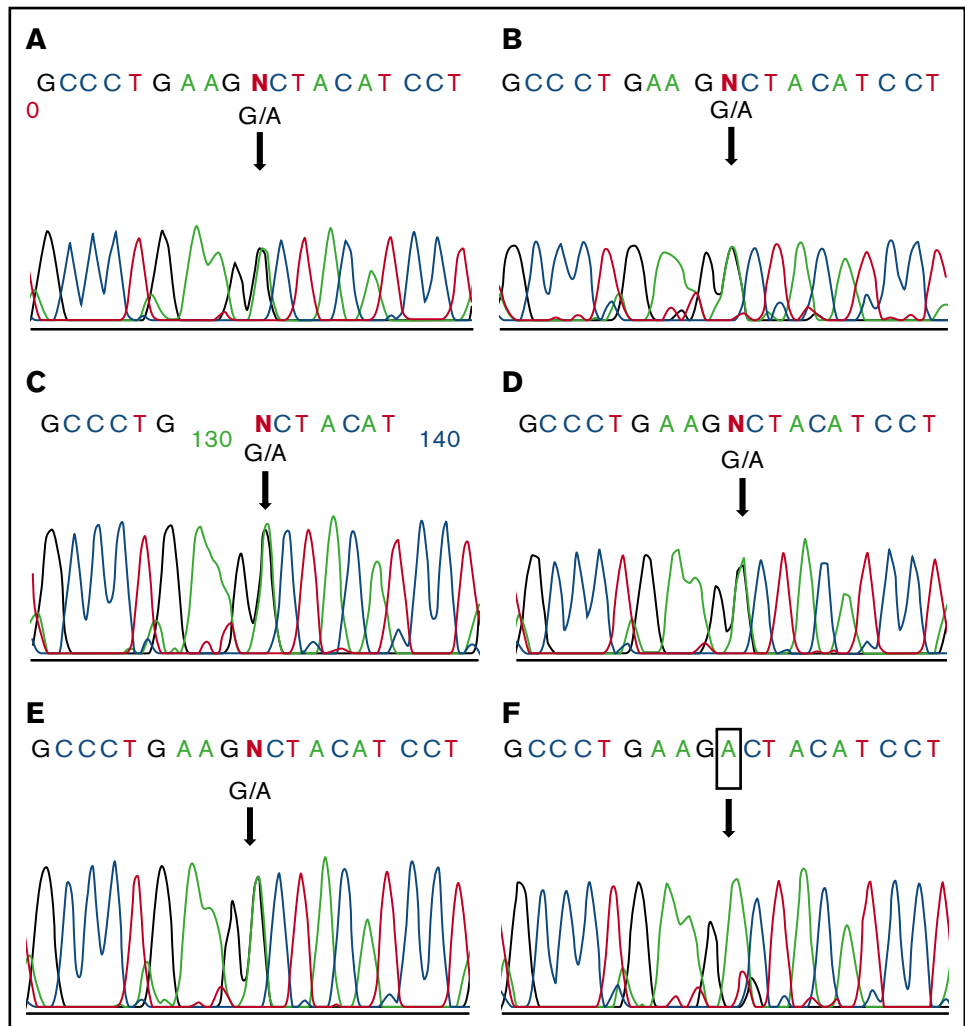
### Expression of recombinant TM proteins

The recombinant TM proteins rhsTM and rhsTM G412D were verified with CBB stain of SDS-PAGE and western blotting. The



**Figure 2. The TM G412D gene expression (RT-PCR products) and protein production in the patient.** (A) The expression of TM G412D mRNA in the PBMCs of the patient (Pt) was detected by RT-PCR and was comparable with that in PBMCs of a healthy control (WT). NC, negative control. (B) The TM G412D protein concentration in the patient's plasma was found to be 17.5 ng/ml using a pAb-ELISA, but was not detected by mAb-ELISA, recognizing the thrombin-binding site (MFTM-4).

**Figure 3. The TM gene sequences of patient's family.** Other family members (A, father; B, mother; C, elder sister; D-E, triplet sisters) were heterozygous for the G1289A mutation of the TM gene, while the patient (F) was homozygous.



relative molecular masses of rhsTM and rhsTM G412D expressed by the yeast expression system as determined by CBB stain after SDS-PAGE were ~97.5 kDa (Figure 4A), which was slightly higher than that of Recomodulin (~95 kDa). rhsTM and rhsTM G412D as well as Recomodulin were detected on western blotting using TM-specific rabbit antibody (Figure 4B), with the reactivity of SDS-treated rhsTM G412D against the antibody evidently decreased compared with that of rhsTM and Recomodulin. Recomodulin, rhsTM, and rhsTM G412D have theoretical molecular weights of 52 171 Da, 55 829 Da, and 55 787 Da, respectively.

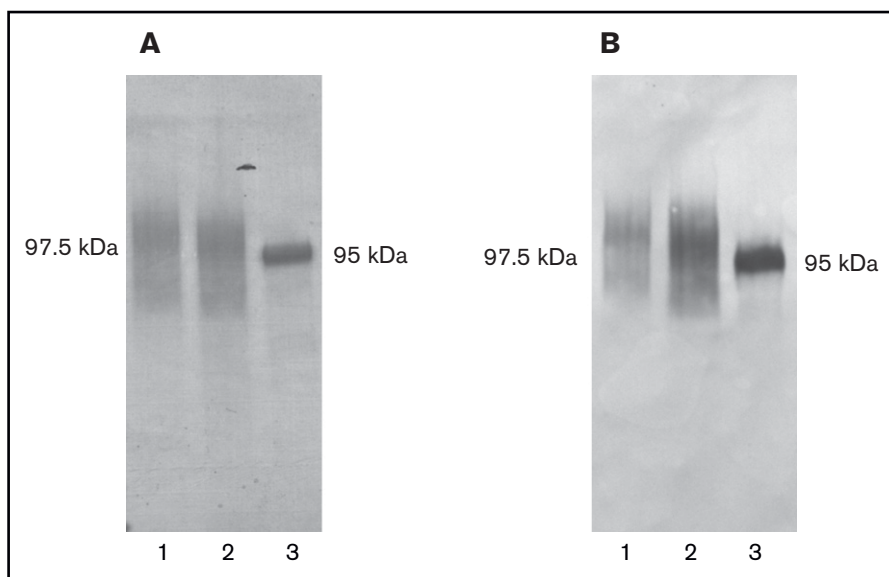
### Functional characterization of rhsTM and rhsTM G412D

To characterize the functional differences between the wild-type TM and the patient's TM, the effects of rhsTM and rhsTM G412D on plasma clotting time were compared. rhsTM significantly ( $P < .01$ ) prolonged the PT, APTT, and TCT in a concentration-dependent manner (Figure 5A-C, respectively), while rhsTM G412D did not (Figure 5D-F, respectively). rhsTM inhibited the thrombin generation in the TGT assay as concentration increased (Figure 6A) but rhsTM G412D did not (Figure 6B). rhsTM as well

as Recomodulin showed a significant cofactor activity that promoted thrombin-catalyzed protein C activation (Figure 6C;  $P < .01$ ), but the cofactor activity of rhsTM G412D was markedly reduced. Based on these data, the cofactor activities of Recomodulin, rhsTM and rhsTM G412D, determined by the previously described method,<sup>21</sup> were calculated to be 121 U/mol, 111 U/mol, and 2.0 U/mol, respectively. Similarly, Recomodulin and rhsTM markedly enhanced the thrombin-catalyzed activation of TAFI, whereas rhsTM G412D did not (Figure 6D). Figure 6E shows that thrombin bound to rhsTM but not to rhsTM G412D, which were immobilized on the solid phase, in proportion to the thrombin concentration. These findings indicate that rhsTM G412D, which cannot bind to thrombin, lost its function as a modulator of thrombin-dependent anticoagulant and antifibrinolytic properties.

### Discussion

TM plays important roles in anticoagulation and anti-inflammation by binding to thrombin. TM has 6 EGF-like domains in the middle of the molecule, among which the EGF4-6 domains are essential for thrombin-mediated protein C activation<sup>20,22</sup> while the EGF3-6 domains are essential for TAFI activation.<sup>23</sup> Biochemical studies<sup>24</sup>

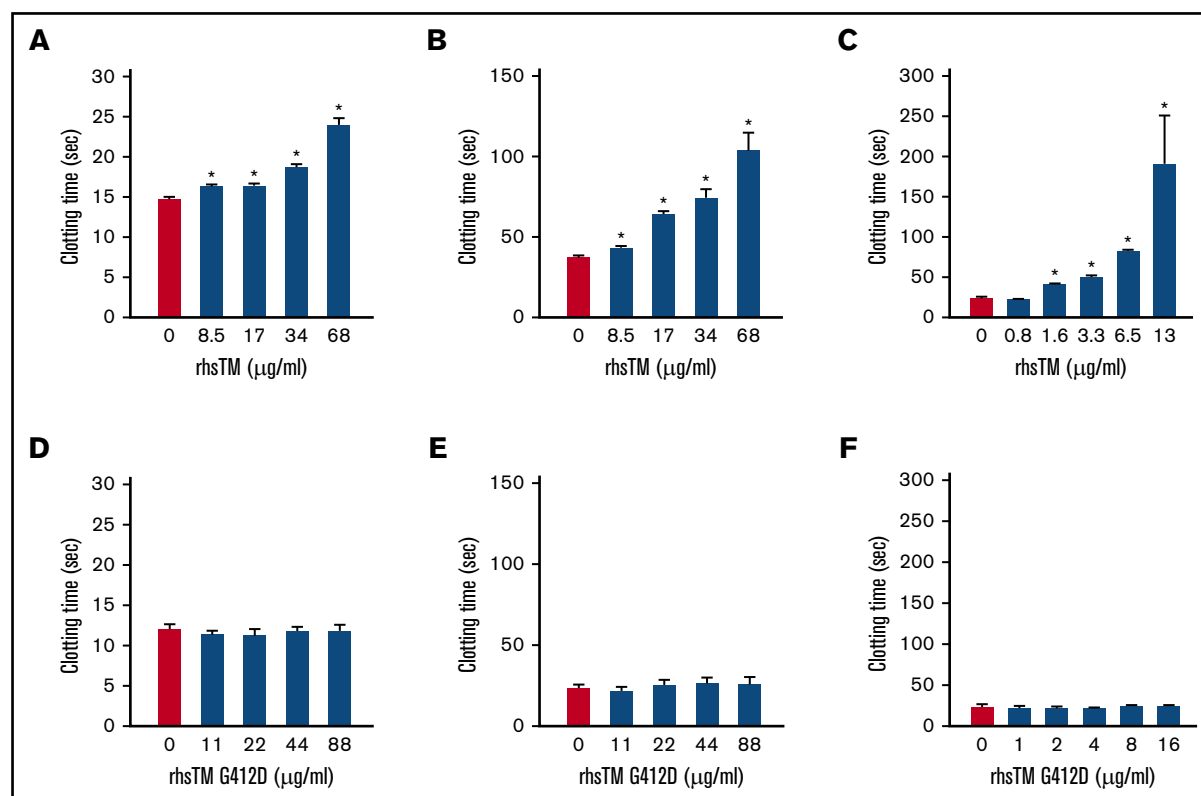


**Figure 4. The expression of recombinant TMs.** (A) SDS-PAGE of recombinant TMs. Each protein (5  $\mu$ g) reduced with 2-mercaptoethanol in the presence of SDS was loaded in each lane, and after electrophoresis, the proteins were stained with CBB. Lane 1, rhsTM G412D; lane 2, rhsTM; and lane 3, Recomodulin. (B) Western blotting of recombinant TMs. Each protein (4  $\mu$ g) reduced with 2-mercaptoethanol in the presence of SDS was loaded in each lane, and after SDS-PAGE the proteins transferred onto the nitrocellulose membrane were detected with antihuman TM polyclonal antibody as described in the text. Lane 1, rhsTM G412D; lane 2, rhsTM; and lane 3, Recomodulin. The molecular weight of recombinant TMs was determined by a comparison with the mobility of commercial marker proteins.

and a crystal structural analysis<sup>25</sup> suggest that EGF5-6 domains serve as a thrombin-binding site.

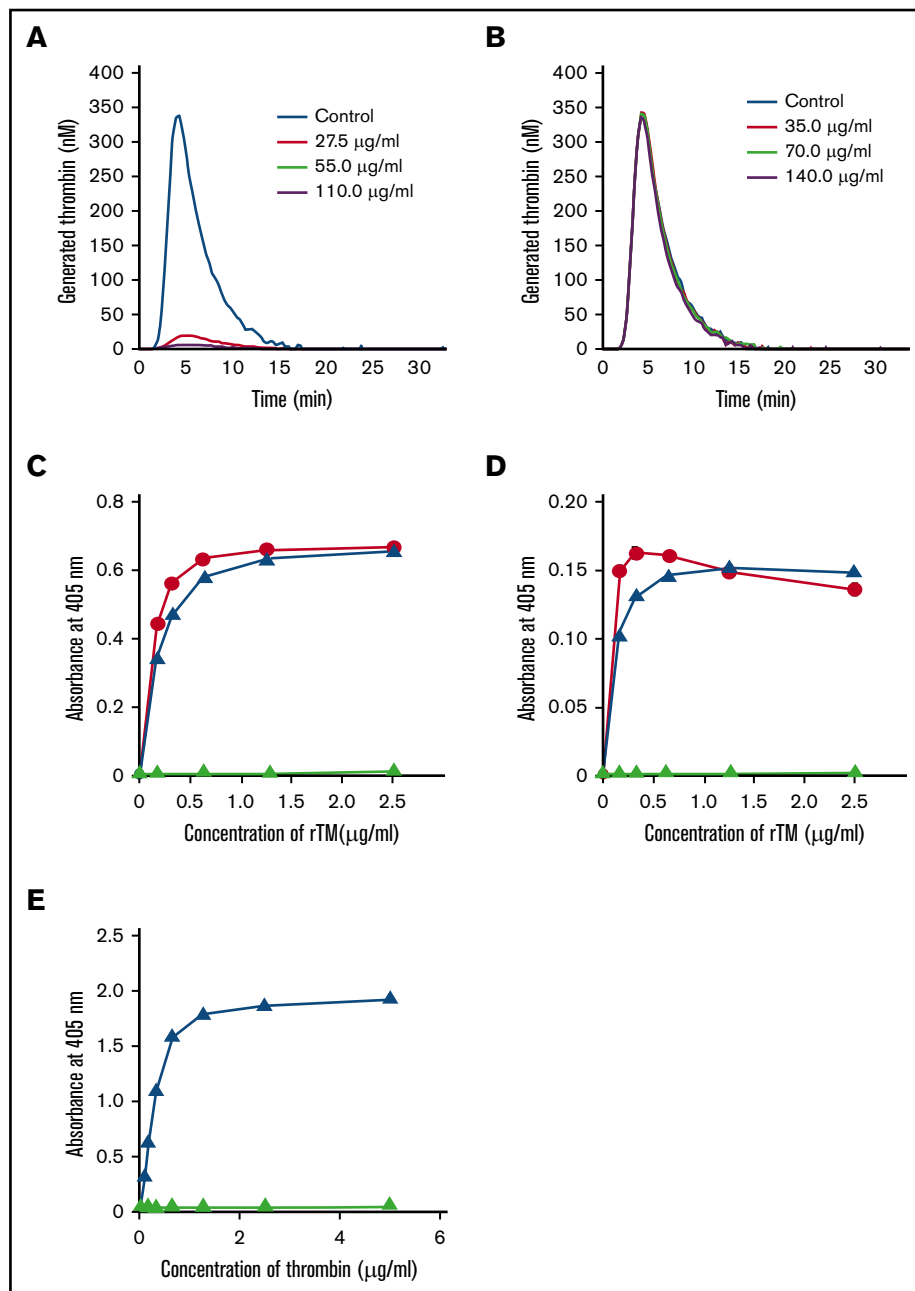
In animal studies, embryos of TM-null mice (TM<sup>-/-</sup>) were dead at 8.5 to 9.5 days of gestation; however, their germinal growth could be achieved if they were isolated at 7.5 days and cultured ex vivo, suggesting that TM is essential for the placental function.<sup>12</sup>

In contrast, while mice with a homozygous Glu404Pro (human Glu387Pro equivalent) mutation in the interdomain loop between the EGF4 and EGF5 domains of TM were born normally, their TM was unable to bind to thrombin and their protein C activation was reduced 1000 times in comparison with those in wild-type mice.<sup>13</sup> Furthermore, fibrin deposition was observed in the lungs, heart, and kidneys of these mice.



**Figure 5. The effects of recombinant TMs on plasma clotting time.** The effects of rhsTM and rhsTM G412D on PT (A,D), APTT (B,E), and TCT (C,F) of human plasma were assessed by adding rhsTM (A-C) or rhsTM G412D (D-F), respectively. The detailed experimental conditions are described in the text. The results are expressed as the mean clotting time (sec)  $\pm$  standard deviation of 3 independent experiments. Significant differences in values between samples with and without recombinant TM are shown as \* $P$  < .01.

**Figure 6. The effects of recombinant TMs on the thrombin generation.** The effects of various concentrations of rhsTM (A) and rhsTM G412D (B) on the TGT were determined using human plasma and a calibrated automated thrombogram system. The effect of rhsTM (blue triangles), rhsTM G412D (green triangles), or Recomodulin (red circles) on thrombin-catalyzed protein C activation (C) and TAFI activation (D) were determined using purified protein C and TAFI, respectively. (E) The thrombin-binding ability of rhsTM (blue triangles) and rhsTM G412D (green triangles) was determined by measuring the amidolytic activity of thrombin bound to recombinant TMs fixed in the microplate wells. The detailed experimental conditions are described in the text.



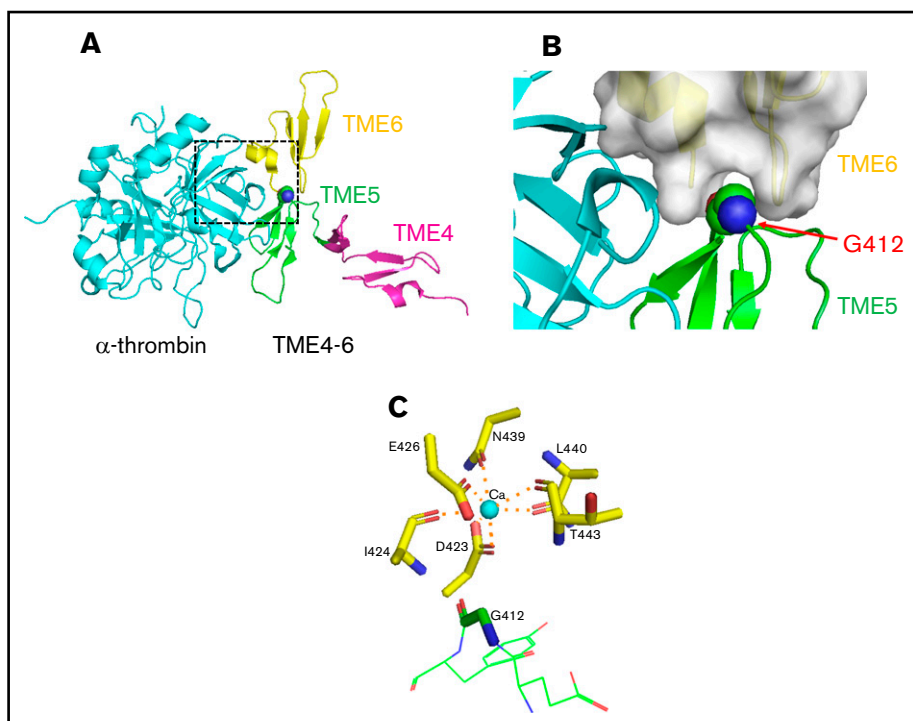
Such phenotypic discrepancy between TM-null mice and those with the mutation in the thrombin-binding site implies that TM is a multifunctional protein and that mutations in the thrombin-binding site can result in abnormalities in the coagulation-fibrinolysis system without affecting fetal development or growth. However, human counterparts of these mice had not been identified.

We demonstrated that the patient examined in this study, whose condition was characterized by recurrent subcutaneous hemorrhage and substantial coagulation-fibrinolysis system abnormalities, had a missense mutation in the EGF5 domain of TM. The mutant TM was unable to bind to the monoclonal anti-TM antibody recognizing the thrombin-binding site, even though it was expressed normally at a level comparable to the healthy control. In vitro assays using

rhsTM protein with the same mutation as the patient examined in this study (rhsTM G412D) demonstrated that the mutant TM was unable to bind to thrombin and activate protein C and TAFI.

From the abovementioned results, all abnormalities found in the coagulation-fibrinolysis system of the patient examined in this study can be explained by functional deficiency of TM, although the patient may not be an exact replica of mice with functional TM deficiency reported by Weiler-Guettler et al,<sup>13</sup> which was associated with a marked decrease of APC and fibrin deposition to many organs.

We investigated why the TM-Nagasaki molecule (rhsTM G412D) was unable to bind to thrombin, based on the analytical findings of the 3-dimensional structure of the cocrystal between epidermal growth factor–like domains of human thrombomodulin (TME)4-6



**Figure 7. Three-dimensional structure of TME4-6 and  $\alpha$ -thrombin complex.** (A) Ribbon models of the complex between  $\alpha$ -thrombin (blue) and TME4-6 (pink, EGF4 domain; green, EGF5 domain; yellow, EGF6 domain). The dashed black box indicates the interaction interface between thrombin and TM as shown in B. (B) The thrombin and TM interaction interface. The surface representation of the EGF6 domain is overlaid with G412 in the EGF5 domain that is shown as spheres (blue, red, and green spheres are nitrogen, oxygen, and carbon atoms, respectively). (C) Stick representation of the calcium-binding residues in the EGF6 domain. The  $\text{Ca}^{2+}$  is shown as a light-blue sphere. The 3-dimensional structure of the thrombin-TM complex was obtained from the database of the Protein Data Bank (PDB; <https://www.rcsb.org/>) using PDB ID:1DX5.26.

(composed of the EGF4-6 domains) and thrombin.<sup>25</sup> Figure 7A shows a state in which thrombin binds to TM via the EGF5-6 domains. Judging from this 3-dimensional structure, G412 in the EGF5 domain does not appear to be located at the binding interface between thrombin and TM but is instead in contact with the EGF6 domain (Figure 7B). It is reasonable to consider that when an Asp mutation occurs in G412, the side chain of the Asp residue affects several amino acid residues of the EGF6 domain and thereby disrupts the 3-dimensional structure of the EGF5-6 domain.  $\text{Ca}^{2+}$  reportedly interacts with each of the amino acids D423, I424, E426, N439, L440, and T443 in the EGF6 domain to form a cluster that stabilizes its structure,<sup>25,26</sup> and these amino acids are reportedly essential for the expression of protein C-activating cofactor activity by thrombin.<sup>27</sup> Since the G412 residue is located near this  $\text{Ca}^{2+}$ -forming cluster (Figure 7C), the G412D mutation may affect the  $\text{Ca}^{2+}$  cluster. We therefore considered that the TM G412D mutation does not directly inhibit the interaction between TM and thrombin but instead changes the conformation of the EGF5-6 domains of TM, resulting in a reduced affinity for thrombin. Furthermore, such conformational changes in the thrombin-binding site may have abolished the binding of the monoclonal anti-TM antibody (MFTM-4)<sup>14</sup> used for the mAb-ELISA to determine plasma TM as a solid-phase antibody that recognizes the thrombin-binding site to TM.

We postulated that the bleeding events in the patient examined in this study followed thrombus formation due to a defect in the TAFI-dependent inhibition of fibrinolysis in the venous system. TM G412D is unable to bind thrombin, leading to a significantly reduced activation of protein C and TAFI. In addition, free thrombin converts fibrinogen to fibrin monomer, greatly increasing fibrin monomer complexes and prothrombin fragments (F1 + F2) and thereby increasing thrombus formation. Free thrombin is also

inhibited by AT-bound heparan sulfate on endothelial cells, and TAT is significantly increased. Furthermore, reduced TAFI activation significantly increases fibrinolysis, such as the FDP and PIC production.

The old cerebral infarction was possibly due to an embolism with a blood clot that had been formed in the venous system and shifted to the arterial current through the foramen ovale during the fetal period. Careful follow-up is critical to learn the long-term prognosis of functional TM deficiency secondary to the mutation in the thrombin-binding site and to clarify how to manage the patient.

In summary, we propose that functional TM deficiency may be a new entity of thrombophilia syndromes, and meticulous analyses of this patient will help us understand the physiological roles of TM, including the role in the coagulation-fibrinolysis system.

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## Authorship

Contribution: G.H., K.S., and M.O. designed research studies; N.T., G.H., J.N., N.A., and T.H. conducted experiments and acquired data; G.H., K.S., and M.O. analyzed data; and M.O., G.H., K.S., and H.M. wrote the manuscript.

Conflict-of-interest disclosure: G.H. is an employee of Asahi Kasei Pharma Corporation. K.S. reports receiving researching funding from Asahi Kasei Pharma Corporation. The remaining authors declare no competing financial interests.

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