Letter to the Editor-in-Chief

Fibrin monomers derived from thrombogenic dysfibrinogenemia, Naples-type variant (BβAla68Thr), showed almost entirely normal polymerization

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Inherited fibrinogen disorders causing quantitative or qualitative alterations in this molecule have been classified phenotypically as hypofibrinogenemia, afibrinogenemia, dysfibrinogenemia, and hypodysfibrinogenemia. As many as 400 cases of inherited fibrinogen disorders have been listed on the GEHT homepage [1]. The clinical manifestations of inherited fibrinogen disorders are very heterogeneous: the absence of symptoms, bleeding, thrombosis, and recurrent miscarriages in women as well as rare cases of fibrinogen storage disease and renal amyloidosis. Approximately 14% of reported dysfibrinogens are associated with thrombosis [2]; however, difficulties are associated with demonstrating that fibrinogen mutations directly cause thrombosis. The homozygous BβAla68Thr mutation, known as Naples I or Milano II [3], which is one of the most common mutations associated with thrombosis, causes markedly defective thrombin binding to its fibrin *in vitro* and an increase in free active thrombin in the circulation [4]. Zhou J *et al.* reported the same mutation in five Chinese patients, two of whom had thrombosis [5].

We recently identified homozygous dysfibrinogenemia, a BβAla68Thr variant, in a 17-year-old Japanese boy who developed superior sagittal sinus thrombosis [6]. We herein examined the thrombin-associated functions of fibrinogen purified from the plasma of this homozygous patient, and compared them with those of fibrinogen from a

heterozygous patient and normal control.

This study was approved by the Ethics Review Board of Shinshu University School of Medicine (approved number 603). After informed consent had been obtained from the propositus and his family members, blood samples were collected for biochemical and genetic analyses. Coagulation tests, DNA sequencing, the purification of plasma fibrinogen, kinetics of fibrinopeptide A (FpA) and fibrinopeptide B (FpB) release, and fibrin polymerization were examined as previously described [7-9], with minor modifications.

The coagulation test results of plasma collected from the propositus, his parents, and two healthy sisters are shown in Table 1. The plasma fibrinogen concentration ratios (the thrombin time method/immunological method) of the propositus and his younger sister were 0.369 and 0.364, respectively, and were lower than those of the others (0.594 ~ 0.671). Moreover, the thrombin clotting times of the propositus' and his younger sister's plasma were 90.0 and 118.0 sec, respectively, and were longer than those of the others (20.6 ~ 24.5). Plasma levels of protein C, protein S, antithrombin, plasminogen lupus anticoagulant, antiphospholipid antibodies, and homocysteine in the propositus were within normal ranges, but were not measured in his younger sister's plasma [6].

A DNA sequence analysis of *FGA*, *FGB*, and *FGG* for the propositus and his

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younger sister revealed a homozygous G>A mutation in exon 2 of FGB (c.292 G>A; NCBI NM_005411), resulting in a substitution of Bβ68Ala (GCT) >Thr (ACT) in the mature protein (or Bß98T with the signal peptide), while the others were heterozygotes for this mutation. This mutation is known as fibrinogen Naples, which causes thrombogenic dysfibrinogenemia in homozygous patients, but not in heterozygous patients.

The rate of thrombin-catalyzed FpA release at an ambient temperature from the propositus' fibrinogen was markedly slower than that from normal control fibrinogen, while that from the father's fibrinogen was slightly slower (**Fig. 1A**). Furthermore, the rate of thrombin-catalyzed FpB release from the propositus' fibrinogen was markedly slower, while that from the father's fibrinogen was slightly slower (**Fig. 1B**). However, the rates of batroxobin-catalyzed FpA release from the propositus' and father's fibrinogen were similar to that from normal control fibrinogen (data not shown).

Representative curves of thrombin-catalyzed fibrin polymerization and fibrin monomers performed at an ambient temperature are shown in **Figure 1C-1F**. The propositus' fibrinogen showed a longer lag period and markedly decreased thrombin-catalyzed fibrin polymerization than the normal control under the conditions of 0.18 mg/mL of fibrinogen and 0.05 U/mL of thrombin. The heterozygous father's

fibrinogen showed a <u>slightly longer lag period and a decreased rate of thrombin-catalyzed fibrin polymerization than the control</u> (**Fig. 1C**). However, batroxobin-catalyzed fibrin polymerization in the propositus and his father was similar to that of the normal control under the conditions of 0.18 mg/mL of fibrinogen and 0.05 U/mL of batroxobin (data not shown).The polymerization of the propositus' fibrinogen was more than two-fold the maximum slope of the reaction curve and there was an absorbance change at 60 minutes at 0.36 mg/dL of fibrinogen, relative to 0.18 mg/dL (**Fig. 1D**). Furthermore, under the condition of 1.0 U/mL of thrombin, the polymerization of the propositus' fibrinogen showed a 16-fold shorter lag period and steeper slope than that under the condition of 0.05 U/mL of thrombin (**Fig. 1E**).

In order to analyze the polymerization ability of homozygous variant fibrinogen, fibrin monomers derived from a thrombin-initiated clot were re-polymerized in pH 7.4 buffer. In contrast to our expectations, fibrin monomers derived from a homozygous variant fibrin clot were re-polymerized in a similar manner to those from the normal control (**Fig. 1F**). Furthermore, in order to examine the marked differences observed in thrombin-catalyzed polymerization, we measured clottability after a 5-hour incubation at 37°C. Clottability was $97.4 \pm 1.3\%$ for normal control fibrinogen. The corresponding values of the propositus' and father's fibrinogen were $87.8 \pm 3.1\%$ ($p < 0.01$) and $84.5 \pm$

2.3% ($p < 0.005$), respectively.

We observed fibrin clots using scanning electron microscopy in order to analyze differences in the ultrastructures of fibrin clots formed at 37°C from the fibrinogen of the normal control, propositus, and his father. Although the density of the bundles of fibrin fibers was less in the order of clots made from fibrinogen of the normal control, the father (heterozygote), and the propositus (homozygote) (photo not shown), fiber diameters were not significantly different between them (data not shown).

We herein identified homozygous dysfibrinogenemia, BβAla68Thr, which manifested as severe thrombosis. A family study revealed one homozygous and three heterozygous members, none of whom exhibited thrombotic complications. The same variant in an asymptomatic heterozygous patient was previously reported as fibrinogen Shizuoka, a resident in the same city as the present propositus (Yonekawa O. *et al.*, 16th Congress of International Society on Thrombosis and Haemostasis, Florence, 1997, PS-2550). Three homozygous patients of the BβAla68Thr fibrinogen family (Naples I or Milano II) manifested thrombosis: two developed strokes at 25 and 21 years of age, respectively, while the other developed DVT after surgery [3]. The markedly defective thrombin binding of homozygous BβAla68Thr fibrin was demonstrated *in vitro* as the thrombosis-inducing mechanism, suggesting an increase in free active thrombin in the

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circulation of the homozygous BβAla68Thr patient [4]. Zhou J *et al.* reported that five

patients from one Chinese family had the same variants; two out of the five patients had

thrombosis, and it was unclear whether two patients were a homozygote or heterozygote

[5].

The homozygous BβAla68Thr variant also showed a markedly prolonged thrombin

clotting time, lower fibrinogen ratio (functional method/immunological method), and

markedly reduced release rate of FpA and FpB by thrombin. In contrast, the

heterozygous BβAla68Thr variant showed a slightly prolonged thrombin clotting time,

slightly lower fibrinogen ratio, and slightly reduced release rate of FpA and FpB by

thrombin. The thrombin-catalyzed fibrin polymerization of homozygous BβAla68Thr

fibrinogen was markedly less than that of the normal control (longer lag period and less

steep slope), whereas fibrin monomers derived from the homozygous variant fibrin clot

were re-polymerized in a similar manner to those from the normal control. The

markedly reduced thrombin-catalyzed fibrin polymerization of homozygous

BβAla68Thr fibrinogen was caused by the markedly delayed release of FpA and FpB.

However, once FpA and FpB were released and fibrin monomers were produced, the

substitution of Thr for BβAla68 did not affect fibrin monomer polymerization. This

phenomenon was markedly different from the aberrant polymerization of fibrin

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monomers derived from the thrombin-catalyzed fibrin of recombinant variant

BβGly15Cys- and BβGly15Ala-fibrinogen [10]. These findings demonstrate that the

BβAla68 residue is not important for the lateral aggregation of desAB fibrin monomer

polymerization.

Homozygous BβAla68Thr fibrinogen showed markedly aberrant

thrombin-catalyzed fibrin formation at an ambient temperature, but also nearly normal

thrombin-induced fibrinogen clottability and clot structures (and fiber diameters) in a

37°C reaction. We speculate that a longer incubation at 37°C may enhance the

thrombin-catalyzed fibrin polymerization of homozygous BβAla68Thr fibrinogen.

Therefore, the fibrin conversion ability of the fibrinogen of homozygous patients may

not be severely reduced *in vivo* and the risk of bleeding may not be very high.

Disclosure of Conflict of Interests

The authors state that they have no conflicts of interest.

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Table 1 Coagulation tests for plasma

	PT (%)	APTT (sec)	Fbg (g/L)		Ratio	TCT (sec)
			functional	immunological		
Proband (Ho)	33.9	43.0	1.38	3.74	0.369	90.0
Father (He)	98.8	31.6	1.87	3.15	0.594	22.9
Mother (He)	105.0	27.5	1.44	2.27	0.634	24.5
Elder sister (He)	100.8	30.0	1.93	2.88	0.671	20.6
Younger sister (Ho)	48.5	33.5	1.00	2.75	0.364	118.0
Normal range	70~130	23~38	1.80~3.50	1.80~3.50	0.80~1.20	10~14

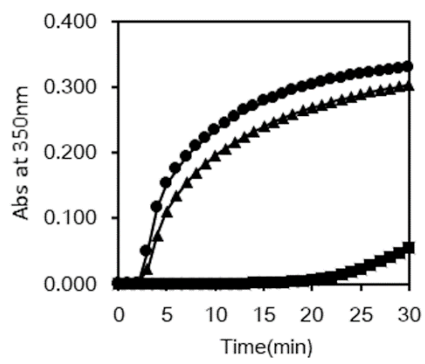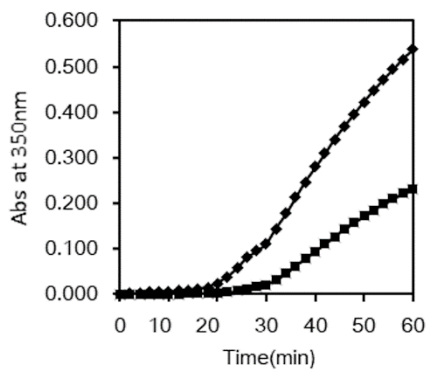Ho: homozygote, He: heterozygote, TCT: thrombin clotting time
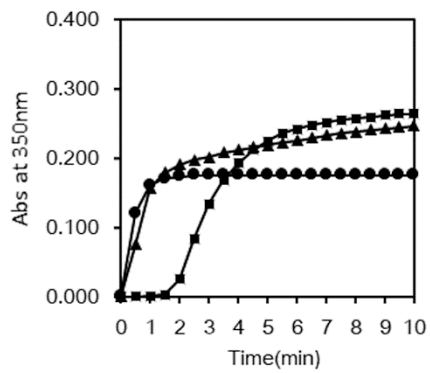
A:FpA release
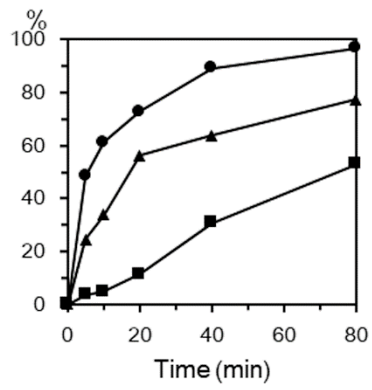
B:FpB release
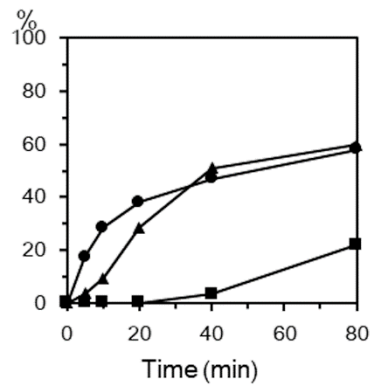
C:Fibrin polymerization

D:Fibrin polymerization

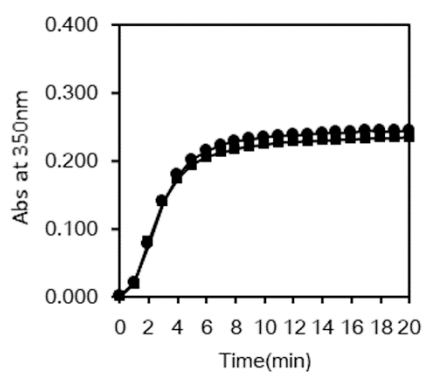E:Fibrin polymerization

F:FM polymerization

Figure legend

Figure 1. Thrombin-catalyzed fibrinopeptide release, fibrin polymerization, and fibrin monomer polymerization. Percent fibrinopeptide release curves from a HPLC assay are shown in Panel **A**: FpA and Panel **B**: FpB with thrombin (0.05 U/ml) catalysis. Reactions were run with the wild-type (●), father's (heterozygote's) (▲), and propositus' (homozygote's) (■) fibrinogen (0.35 mg/ml) at an ambient temperature in HBS. The 100% release of FpA and FpB was catalyzed with 3.9 U/mL of thrombin at 37°C for 80 minutes.

The thrombin-catalyzed polymerization of fibrinogen (0.18 mg/ml) was initiated with 0.05 U/mL of thrombin at an ambient temperature (Panel **C**). Panel **D**: The thrombin-catalyzed polymerization of the propositus' fibrinogen, 0.18 (■) or 0.36 mg/ml (◆), was initiated with 0.05 U/mL of thrombin. Panel **E**: The thrombin-catalyzed polymerization of fibrinogen (0.18 mg/ml) was initiated with 1.0 U/mL of thrombin. Panel **F**: A total of 0.1 mg/mL of fibrin monomers (derived from the thrombin-initiated clot) was re-polymerized in pH 7.4 buffer (normal control and the homozygous propositus). Representative turbidity curves from triplicate experiments are shown for the normal control (●), father's (heterozygote) (▲), and propositus's (homozygote) (■) fibrinogen (Panels C–F).