Development of a new modified Bethesda method for coagulation inhibitors: the Osaka modified Bethesda method

Sumiko Torita[a], Etsuji Suehisa[a], Tomio Kawasaki[b], Masayuki Toku[a], Emi Takeo[a], Yoshiaki Tomiyama[c], Sumiyuki Nishida[d] and Yoh Hidaka[a]

The Nijmegen assay for the factor VIII (F-VIII) inhibitor is recommended by the International Society on Thrombosis and Haemostasis/Scientific and Standardization Committee. However, due to cumbersome and complicated preprocessing, it is presently difficult to introduce this assay into hospital laboratories. We used buffered plasma that was made by addition of 1 volume of 1 mol/l HEPES buffer at pH 7.35 to 9 volumes of plasma to form the test samples. The inhibitor titer was calculated by the remaining rate of F-VIII coagulation activity (F-VIII:C), using the ratio of actual value to the theoretical value. Five hundred microliters of the buffered test plasma and the control (30 mmol/l HEPES buffered saline at pH 7.35) were each mixed with equal volumes (500 μl) of normal pooled plasma in a test tube (11 mm internal diameter and 6.5 ml volume capacity), and incubated at 37°C for 2 h. In our modified Bethesda method, there were no significant changes in pH and F-VIII:C of control and test mixtures after incubation tests for stability. With the modified method, the inhibitor titers (mean, SD) from examining three hemophilia A plasma samples (F-VIII:C, <1–3%) and 40 normal samples (F-VIII:C, 34.5–168.3%) were 0.032, 0.057 and -0.009, 0.057, respectively. By our method, the F-VIII inhibitor titer of type I inhibitor-positive samples was higher than the Nijmegen method, and for type II inhibitor-positive samples, the titer was similar. We believe that our method can be applied to not only the type I inhibitor, but also to assays of type II inhibitor, without cumbersome and complicated preprocessing.
Blood Coagul Fibrinolysis 22:185–189 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Blood Coagulation and Fibrinolysis 2011, 22:185–189

Keywords: Bethesda assay, coagulation inhibitor, pH

[a]Laboratory for Clinical Investigation, Osaka University Hospital, [b]Cardiovascular Surgery, [c]Hematology and Oncology and [d]Cancer Immunotherapy, Osaka University Graduate School of Medicine, Osaka, Japan

Correspondence to Sumiko Torita, BHSc, Laboratory for Clinical Investigation, Osaka University Hospital, 2-15 Yamada-oka, Suita City, Osaka 565-0871, Japan
Tel: +81 06 6879 6655; fax: +81 06 6879 6657;
e-mail: torita@hp-lab.med.osaka-u.ac.jp

Received 14 May 2010 **Revised** 22 November 2010
Accepted 1 December 2010

Introduction

The development of inhibitors against factor VIII (F-VIII) can appear in patients with hemophilia A who are treated with F-VIII replacement therapy, and also spontaneously in nonhemophilia A. The incidence of inhibitor development against F-VIII is highest in patients with hemophilia A (approximately 30%) in response to infusions of F-VIII [1–4]. An assay of inhibitors against F-VIII was reported by Kasper *et al.* [5] as the Bethesda method in 1975, and introduced a measurement of F-VIII inhibitors by using normal pooled plasma (NPP) as the F-VIII source in a 1:1 mix with patient plasma and imidazole buffer as the control sample.

The Bethesda assay is widely used to monitor the development and progress of the F-VIII inhibitor, but it lacks specificity in the lower range resulting in false-positives due to F-VIII:C in the plasma that is compromised by pH elevation. The specificity and sensitivity of the assay was further improved in the Nijmegen assay [6], and the assay is recommended by the International Society of Thrombosis and Haemostasis Factor VIII/IX Scientific Subcommittee [7]. However, due to cumbersome and complicated preprocessing, including a heating treatment of the plasma for inactivation of coagulation factors and buffering of NPP, it is presently difficult to carry out the assay in hospital laboratories.

For detection of the F-VIII inhibitor, there is a need for a simple, sensitive, and reproducible assay. The aim of this study is development of a modified Bethesda assay to enhance the convenience of F-VIII inhibitor detection.

Materials and methods

Plasma preparation

Venous blood samples were obtained by venipuncture with the use of a tourniquet. Blood was drawn into Vacutainer tubes containing 3.2% (w/v) trisodium citrate (0.129 mol/l; 9:1, v/v). Platelet-poor plasma was prepared by centrifugation at 2800*g* for 10 min. NPP used in the study was derived from blood collected from 40 normal individuals. Each individual plasma sample was screened for activated partial thromboplastin time (APTT) and prothrombin time (PT).

Test samples

Three hemophilia A samples and six F-VIII inhibitor-positive samples were measured by both our modified

0957-5235 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/MBC.0b013e32834356d2

method and the Nijmegen modified method, for the evaluation of the inhibitor activity. Four of the six inhibitor-positive plasmas were made by adding a portion of commercially available inhibitor-positive plasma (George King Bio Medical, Overland Park, Kansas, USA) to hemophilia A plasma. The remaining two of the six inhibitor-positive plasmas were made by adding a portion of F-VIII type II inhibitor-positive patient plasma to normal plasma.

Factor VIII:C assay

The F-VIII:C assay was measured in a one-stage APTT-based clotting assay on a Coagrex 800 (Sysmex, Kobe, Japan). APTT reagent (Thrombocheck APTT-SLA; Sysmex) and F-VIII-deficient plasma were obtained from Sysmex (Sysmex).

pH measurement

All pH measurements were carried out with a PHM-210 standard pH meter (Radiometer, Copenhagen, Denmark).

Inhibitor assay

Nijmegen modified Bethesda assay

The Nijmegen modified Bethesda assay was performed as described previously [8].

We used the supernatant from centrifugation at 4000*g* for 5 min after heating at 58°C for 90 min as test plasma and control plasma. Heated test and control plasma were each mixed with equal volumes of 0.1 mol/l imidazole-buffered NPP at pH 7.4 and incubated at 37°C for 2 h. Thereafter, F-VIII:C in both test and control mixture was measured by a one-stage clotting assay. The remaining F-VIII:C is expressed as the relative percentage of F-VIII:C in the test mixture compared with the control mixture. One Nijmegen Bethesda unit (NBU/ml) is defined as the amount of inhibitor that reduces the remaining F-VIII:C to 50% after 2 h of incubation. The inhibitor activity was calculated in NBU/ml from a semi-logarithmic plot representing the correlation between the remaining F-VIII:C (logarithmic) and inhibitor activity (linear).

Our modified Bethesda assay

We used buffered plasma that was made by addition of 1 volume of 1 mol/l HEPES buffer at pH 7.35 to 9 volumes of plasma as the test samples. Buffered test plasma and 30 mmol/l HEPES buffer at pH 7.35 as control were each mixed with equal volumes of NPP, and incubated at 37°C for 2 h.

To avoid the influence of F-VIII:C in test plasma in the Bethesda assay, the remaining F-VIII:C was calculated using a ratio of the theoretical value and actual value of both the test and control mixture. The remaining F-VIII:C of our method is expressed as the relative percentage of the F-VIII:C ratio (actual value/theoretical value) of the test mixture compared with control mixture. Just like the classical Bethesda method, one modified Bethesda unit (BU/ml) in this method is defined as the amount of inhibitor that reduces the remaining F-VIII:C to 50% after 2 h of incubation. The inhibitor activity was calculated in BU/ml from a semi-logarithmic plot representing the correlation between the remaining percentage of F-VIII:C, calculated from the F-VIII:C ratio, and inhibitor activity. The details of the calculation are as follows.

$$Theoretical\ value\ (Remaining\ factor\ activity) = (NPP\ F\text{-}VIII:C + patient\ F\text{-}VIII:C)/2$$

$$Remaining\ F\text{-}VIII:C\ Ratio(RFR) = Actual\ value/Theoretical\ value$$

$$Percent\ of\ remaining\ F\text{-}VIII:C\ (Z) = (Test\ RFR/Control\ RFR) \times 100$$

$$F\text{-}VIII\ inhibitor\ titer\ (BU/ml) = (\log Z\text{-}2)/-\log 2$$

When an inhibitor titer was more than 0 BU/ml, the value was corrected by dilution ratio (x1.1) for buffering sample. Also, when an inhibitor titer was more than 2 BU/ml, the sample was diluted by 30 mmol/l HEPES-buffered saline and was retested, and then corrected using the dilution ratio.

Statistical analysis

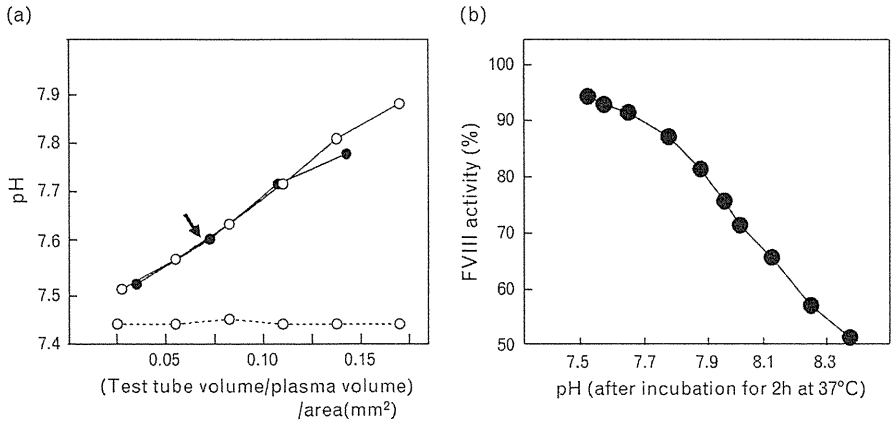
Between-group differences of normal individuals for the inhibitor titer of our modified Bethesda method were analyzed by the Mann–Whitney *U*-test. A *P* value of less than 0.05 was considered statistically significant.

Results

Relationship of tube size and plasma volume to pH

To decide the volume of the reaction mixture, we examined the relationship between pH and the volume of the reaction mixture after incubation at 37°C for 2 h. Each volume of NPP, which was buffered by HEPES buffer at pH 7.35 (HEPES final concentration 50 mmol/l), was added to tubes of two different sizes (11 mm internal diameter and 6.5 ml volume capacity, 14 mm internal diameter and 13 ml volume capacity), capped, and then the pH was measured after incubation at 37°C for 2 h. The relationship between pH and the ratio (test tube volume/plasma volume) per contact area between plasma and the air is shown in Fig. 1a. F-VIII:C decreased with elevation in pH (Fig. 1b). The pH rose with smaller amounts of plasma. On the other hand, when air was not in contact with plasma samples, the pH remained static. This result indicated that tube volume, tube internal diameter, and plasma volume affected the pH elevation in plasma after incubation at 37°C for 2 h. When 1 ml of reaction mixture was added to a test tube of 11 mm

Fig. 1



Relationship between tube size and plasma volume in relation to pH and F-VIII:C. (a) The relationship between pH and the ratio (test tube volume/plasma volume) per contact area between plasma and the air. (b) The relationship between pH and F-VIII:C. Test sample used was buffered normal plasma (normal plasma 9.5 ml + HEPES buffer 0.5 ml). Samples (ml) were added to 14 mm internal diameter (○) or 11 mm internal diameter (●) test tubes. pH and F-VIII:C were measured after incubation at 37°C for 2 h. The dotted line represents a mixture that was sealed from the air. The arrow represents the pH when 1 ml of reaction mixture was added to a test tube of 11 mm internal diameter and 6.5 ml volume.

internal diameter and 6.5 ml volume, the pH of the control and test mixture after incubation was 7.53 and 7.59, respectively. To avoid pH elevation, therefore, we carried out this study on the condition that 1 ml of reaction mixture was added to test tubes of 11 mm internal diameter and 6.5 ml volume capacity.

Stability of factor VIII:C in buffered sample plasma

To confirm the stability of F-VIII:C in the reaction mixture after incubation at 37°C for 2 h, the remaining F-VIII:C was measured by both the Nijmegen and our methods in five normal samples that had 61.8, 62.7, 94, 98.4, and 106.5% of F-VIII:C. The natural reduction of F-VIII:C after incubation was calculated using the remaining F-VIII:C ratio (RFR) of the actual value to the theoretical value of F-VIII:C in the mixture. According to our method, the remaining F-VIII:C in the test and control mixtures was higher than the activity measured by the Nijmegen method (RFR mean ±SD; 0.975 ± 0.040 and 0.997 ± 0.014 by our method, 0.817 ± 0.054 and 0.780 ± 0.018 by the Nijmegen method, respectively). The reduction rate of F-VIII:C by the Nijmegen method was about 20% higher in comparison to our method (Fig. 2).

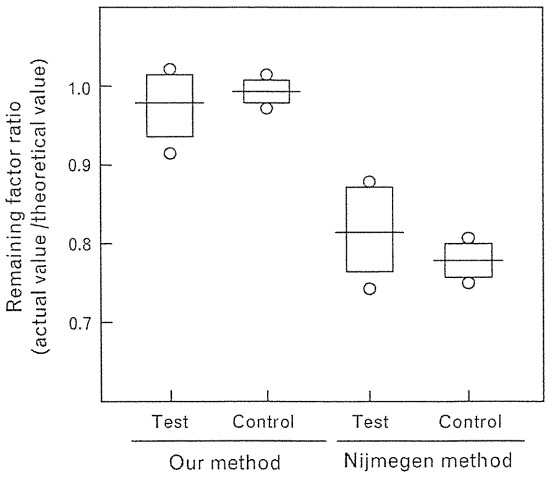
Sample measurement for inhibitor detection

We compared our method with the Nijmegen method in three hemophilia A samples, four type I F-VIII inhibitor-positive plasmas, and two type II F-VIII inhibitor-positive plasmas. There was no difference in the inhibitor titer for hemophilia A samples measured with both methods. By our method, the F-VIII inhibitor titer of type I inhibitor-positive samples was higher than the Nijmegen method, and for type II inhibitor-positive samples, the titer was similar (Table 1).

Measurement of normal individuals

To decide a cut-off value by this method, we measured plasma of 40 normal individuals who had 34.5–168.3% of F-VIII:C. The inhibitor titers (mean, SD; BU/ml) of normal individuals were −0.0093 and 0.0569, respectively.

Fig. 2



Stability of F-VIII activity in our modified method and the Nijmegen method. The F-VIII:C of normal pooled plasma (NPP) and imidazole buffered NPP were 100 and 87.3%, respectively. The F-VIII:C of test plasma was 106.5, 98.4, 62.7, and 61.8%. Stability is represented by the remaining factor ratio (RFR). Solid lines show the mean, boxes show the mean ± SD, and open circles show the maximum and minimum values.

Table 1 Comparison of measurement values by our method and the Nijmegen method

		Modified Bethesda (BU/ml)	
Sample	F-VIII:C (%)	Our method	Nijmegen
Hemophilia A			
1	0.5	−0.034	−0.036
2	0.6	0.064	0.037
3	3.0	0.065	0.088
Type I inhibitor			
4	0.6	1.114	0.754
5	1.1	0.436	0.169
6	2.4	0.216	0.034
7	<0.5	1.884	1.667
Type II inhibitor			
8	58.2	7.546[a]	8.024[a]
9	52.3	3.333[b]	3.580[b]

[a]Value represents eight times dilution. [b]Value represents four times dilution.

Furthermore, when their samples were divided into three groups according to plasma F-VIII:C (F-VIII:C, <50, 50–100, and >100%), the inhibitor titers were not significantly different between the three groups. This result suggests that our modified method is not affected by the quantity of individual F-VIII:C (Table 2).

Reproducibility of measured results in the low titer of factor VIII inhibitor

To determine the accuracy of our method in the low F-VIII inhibitor titer, different amounts of F-VIII inhibitor plasma were added to the heated plasma (F-VIII: C 1.0%>), and reproducibility of F-VIII inhibitor was measured. In these experiments, when these samples were measured five times, the coefficient of variation was 3.28 (mean: 1.07 BU/ml), 4.10 (mean: 0.50 BU/ml), and 12.53 (mean: 0.27 BU/ml).

Discussion

Quantification of the F-VIII inhibitor has an important role in the management of patients with hemophilia A and acquired hemophilia A. An interlaboratory survey of F-VIII inhibitor assay has been conducted by the European Concerted Action on Thrombophilia Foundation in conjunction with the North American Specialized Coagulation Laboratory Association in 2006 and 2007, and reported that the coefficient of variation was high (30–42%) for inhibitor-positive samples [9]. With regard to plasma pH levels after 2 h incubation at 37°C, we identified a relationship of pH elevation between plasma volume and test tube volume, and specifically that pH became elevated with smaller amounts of plasma in test tube. pH elevation can be prevented with smaller amounts of plasma when a smaller volume test tube is used. It is thought that this pH elevation during incubation is caused by drawdown of CO_2 partial pressure by the plasma. In the Bethesda or Nijmegen assay method, the authors described a one-to-one relation between NPP (or buffered NPP) and patient plasma in a mixture; however, they provided no information about test tube volume and plasma volume [5,6]. This high coefficient of variation may be caused by variations of test tube volume and plasma volume during incubation.

Table 2 Measurement values of normal individuals by our modified method

		FVIII:C (%)			Inhibitor (BU/ml)	
		Mean	SD	Range	Mean	SD
<50	(n = 4)	37.9	4.52	34.5–44.5	−0.0235	0.0598
50–100	(n = 19)	79.4	13.91	52.3–95.5	−0.0033	0.0485
>100	(n = 17)	125.1	22.63	100.0–168.3	−0.0127	0.0669
Total	(n = 40)	94.7	33.90	34.5–168.3	−0.0093	0.0569

In the present study, the F-VIII inhibitor titer for the inhibitor-positive samples was higher by our method than the Nijmegen method, although there was no difference between the two methods for inhibitor-negative hemophilia A samples. Verbruggen [8] described in a meeting report that heating of test and control plasma at 58°C for 90 min inactivated all clotting factors, whereas immunoglobulins were heat-resistant leaving the inhibitor data unchanged. However, when heated at high temperature or long periods, immunoglobulin G (IgG) in solution aggregates and results in insoluble precipitates [10]. For this reason, there is a possibility that part of the IgG polymer disappeared by centrifugation after processing of the heat-treated plasma.

F-VIII inhibitors can be divided into either type I or type II. Type I inhibitors, mostly appearing as alloantibodies in F-VIII-treated hemophiliac patients, are able to completely inactivate F-VIII:C at high plasma concentrations. Type II inhibitors, which are frequently autologous antibodies, are unable to completely inactivate F-VIII:C, even at the maximum antibody concentration [11]. Native F-VIII:C in patient plasma affects the inhibitor assay by increasing the remaining factor activity after incubation with NPP, thus leading to a falsely low-inhibitor titer. However, our method can be applied to not only the type I inhibitor but also the type II inhibitor assay. This is because the inhibitor titer is calculated by the remaining rate of F-VIII:C in the reaction mixture using the ratio of actual and theoretical values, and because cumbersome preprocessing, including heat treatments of samples and NPP and buffering of NPP are not required.

We believe that our method can be applied to not only the type I inhibitor, but also to assays of type II inhibitor, without cumbersome and complicated preprocessing. However, data accumulation of patient samples with F-VIII inhibitor and a cross-laboratory study may be necessary to evaluate utility of our method.

Acknowledgement

The present work was supported by a Health Labour Sciences Research Grant (Research on Measures

for Intractable Diseases) from the Ministry of Health, Labour and Welfare. We thank Mr Masahiko Matsui for useful discussion about the elevating pH phenomenon.

The authors state that they have no conflict of interest.

References

1 Lusher JM, Arkin S, Abildgaard CF, Schwartz RS. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy, and development of inhibitors. Kogenate Previously Untreated Patient Study Group. *N Engl J Med* 1993; **328**:453–459.

2 Bray GL, Gomperts ED, Courter S, Gruppo R, Gordon EM, Manco-Johnson M, *et al.* A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. The Recombinate Study Group. *Blood* 1994; **83**:2428–2435.

3 Kreuz W, Ettingshausen CE, Zyschka A, Oldenburg J, Saguer IM, Ehrenforth S, *et al.* Inhibitor development in previously untreated patients with hemophilia A: a prospective long-term follow-up comparing plasma-derived and recombinant products. *Semin Thromb Hemost* 2002; **28**:285–290.

4 Lusher JM, Lee CA, Kessler CM, Bedrosian CL. The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. *Haemophilia* 2003; **9**:38–49.

5 Kasper C, Aledort L, Counts R, Edson R, Fratantoni J, Green D, *et al.* A more uniform measurement of factor VIII inhibitors [letter]. *Thromb Diath Haemorrh* 1975; **34**:869–872.

6 Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost* 1995; **73**:247–251.

7 Giles AR, Verbruggen B, Rivard GE, Teitel J, Walker I. A detailed comparison of the performance of the standard versus the Nijmegen modification of the Bethesda assay in detecting factor VIII:C inhibitors in the haemophilia A population of Canada. Association of Hemophilia Centre Directors of Canada. Factor VIII/IX Subcommittee of Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis. *Thromb Haemost* 1998; **79**:872–875.

8 Verbruggen B. Diagnosis and quantification of factor VIII inhibitors. *Haemophilia* 2010; **16**:20–24.

9 Peerscke EI, Castellone DD, Ledford-Kraemer M, Van Cott EM, Meijer P. Laboratory assessment of factor VIII inhibitor titer: the North American Specialized Coagulation Laboratory Association Experience. *Am J Clin Pathol* 2009; **131**:552–558.

10 Oreskes I, Mandel D. Size fractionation of thermal aggregates of immunoglobulin G. *Anal Biochem* 1983; **134**:199–204.

11 Gawryl MS, Hoyer LW. Inactivation of factor VIII coagulant activity by two different types of human antibodies. *Blood* 1982; **60**:1103–1109.

Int J Hematol (2011) 93:329–335
DOI 10.1007/s12185-011-0791-1

ORIGINAL ARTICLE

Epidemiology of primary immune thrombocytopenia in children and adults in Japan: a population-based study and literature review

Yoshiyuki Kurata · Kingo Fujimura · Masataka Kuwana · Yoshiaki Tomiyama · Mitsuru Murata

Received: 23 November 2010 / Revised: 9 February 2011 / Accepted: 9 February 2011 / Published online: 24 February 2011

Abstract The epidemiology of primary immune thrombocytopenia (ITP) is not well-characterized in the general population. Most published studies, which have included relatively small numbers of ITP patients, have been conducted in England or Scandinavian countries. No epidemiologic data from Asian countries have been published. This study describes the epidemiology of ITP in a Japanese population. We analyzed the database registry of the Ministry of Health, Labour, and Welfare of Japan, and extracted newly diagnosed acute and chronic ITP patients with a platelet count of $<100 \times 10^9$/L. From 2004 to 2007, 7,774 cases of ITP were reported, giving an overall incidence of 2.16/100,000/year. The incidence differed greatly between males and females, being 1.72 and 2.58, respectively. The median age of the total affected population was 56 years old. In male patients, there was a striking preponderance of boys below 4 years and a very high peak among those aged 75–89 years. In female patients, the number of ITP patients appeared to show a trimodal distribution by age, with the first peak representing patients below 4 years, the second peak those aged 20–34 years, and the third peak those aged 50–89 years. In conclusion, the incidence of ITP in Japan is not markedly different from that of European countries studied to date. This population-based study reveals that, contrary to previously published studies, the maximum age-specific incidence is in the eighth decade.

Keywords ITP · Epidemiology · Incidence

Y. Kurata (✉)
Department of Human Welfare, Shitennoji University,
3-2-1 Gakuenmae, Habikino, Osaka 583-8501, Japan
e-mail: ykurata@shitennoji.ac.jp

K. Fujimura
Laboratory of Clinicopathological Therapeutics,
Faculty of Pharmaceutical Science,
Hiroshima International University, Hiroshima, Japan

M. Kuwana
Division of Rheumatology, Department of Internal Medicine,
Keio University School of Medicine, Tokyo, Japan

Y. Tomiyama
Department of Blood Transfusion,
Osaka University Hospital, Suita, Japan

M. Murata
Department of Laboratory Medicine,
Keio University School of Medicine, Tokyo, Japan

1 Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune bleeding disorder in which antiplatelet autoantibodies bind to antigens on the surface of platelets and cause accelerated destruction [1]. Impaired platelet production may also contribute to the low platelet counts [2].

There are only limited data on the incidence of ITP [3–12]. Published studies describe a relatively small number of ITP patients.

We aim to update earlier estimates of the incidence of this disease, as these are essential in designing treatment trials and planning services for the patient population. In the present study, we report up-to-date estimates of the incidence of ITP using the large database of the Ministry of Health, Labour, and Welfare of Japan.

2 Materials and methods

2.1 Patients

The diagnosis of ITP was based on thrombocytopenia (platelet count less than 100×10^9/L), normal or increased

Table 1 Overall and sex-specific incidence of child and adult ITP

	Overall		Childhood		Adult	
	Number of patients	Incidence	Number of patients	Incidence	Number of patients	Incidence
Overall ITP	7,774	2.16	929	1.91	6,845	2.20
Male	3,043	1.72	505	2.01	2,538	1.68
Female	4,731	2.58	424	1.79	4,307	2.69

bone marrow megakaryocytes without morphologic evidence of dysplasia, and no secondary immune or non-immune disease that could account for the thrombocytopenic state [13]. Acute and chronic ITP patients were included in this analysis.

2.2 Health care system for intractable disease in Japan

In Japan, ITP is considered to be an intractable disease and treated as a specified disease. Specified diseases are subsidized partly or totally based on the severity of the disease by public expense. A physician who newly diagnoses and treats an ITP patient issues a medical certificate and an application form for the recognition of the specified disease, which certifies that the patient is suffering from ITP and describes detailed clinical information. The patient applies for the recognition of the specified disease to the Department of Health and Medical Care of the regional prefecture through the regional public health center. Before the application form is registered in the centralized database, the form undergoes a series of quality checks, such as the accuracy of diagnosis according to the criteria mentioned above. Thereafter, the data are sent from the Department of Health and Medical Care of the regional prefecture to the database of the Ministry of Health, Labour, and Welfare of Japan.

2.3 Data analyses

We analyzed the database for the years 2004–2007. The database includes details of patient characteristics, hemorrhagic symptoms, prescription information, and laboratory tests. Population information was obtained from the census of the Ministry of Internal Affairs and Communications. The population included in this study comprised an average of 90.0 million inhabitants, corresponding to 71% of the total Japanese population.

ITP patients were divided by age into those with child and adult ITP. Child ITP was that in patients below 14 years.

Comparisons of therapies and platelet counts were performed using the χ^2 test with Yates's correction. Significance was defined as a probability value of less than 0.05.

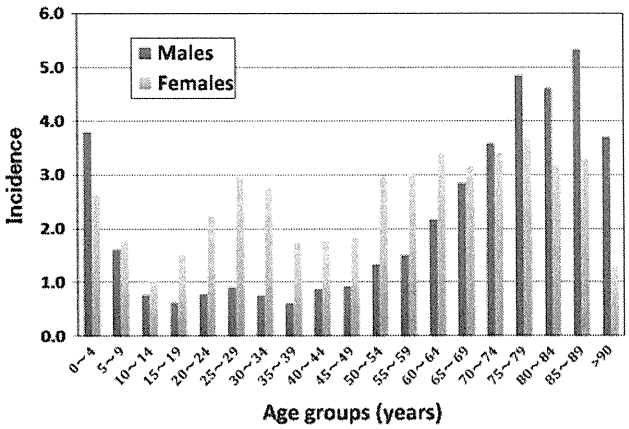


Fig. 1 Age/gender-specific incidence of ITP per 100,000 population

3 Results

3.1 Incidence

There were 7,774 patients in the database, and they consisted of 4,731 females (61%) and 3,043 males (39%) in the years 2004–2007 (Table 1). The annual incidence was 2.16 per 100,000. The incidence differed greatly for males and females. The incidence in females was 2.58, while that in males was 1.72. ITP patients were divided into those with child and adult ITP. The 7,774 patients consisted of 929 with child ITP (females 424; males 505), and 6,845 with adult ITP (females 4,307; males 2,538). The incidence of child ITP was 1.91 (females 1.79; males 2.01), and that of adult ITP was 2.20 (females 2.69; males 1.68).

3.2 Age and sex distribution

The median age of the entire population was 56 years (females 54 years; males 60 years). Figure 1 shows the age/gender-specific incidence, and Fig. 2 shows the age and sex distribution of ITP patients.

In male patients, there was a marked preponderance of boys among newborns and infants below 4 years, after which the incidence fell to a low level between 10 and 49 years old, thereafter rising gradually with increasing age. The highest peak of the incidence was among those aged 75–89 years (Fig. 1), but the peak of the number of patients moved to those aged 65–79 years (Fig. 2). Male

patients at 15–49 years old comprised 21%, while patients over 50 years old made up 62%, meaning that the rate in aged patients was 3.0 times higher than in those of a younger age.

In female patients, the age-specific distribution of ITP patients appeared to have a trimodal distribution, with the first peak observed below 4 years, the second among those aged 20–34 years, and the third peak among those aged 50–89 years (Fig. 1). On the other hand, the highest peak of the number of patients was among those aged 55–59 years (Fig. 2). Female patients aged 15–49 years old comprised 35%, while patients over 50 years old made up 56%.

The number of ITP patients differed greatly between males and females. The F/M ratio in children below 4 years was 0.69. The incidence of ITP among boys below 4 years was higher compared to girls. However, for children in the older age groups, the pattern was reversed, with a lower incidence among boys compared to girls. In patients aged 15–49 and over 50 years old, the F/M ratios were 2.62 and 1.39, respectively. The sex difference was eliminated in patients at 65–74 years old, and then there was a marked predominance of male patients.

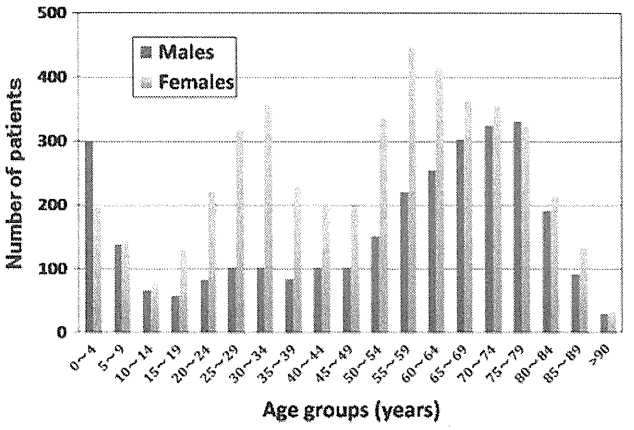


Fig. 2 Age and sex distribution of ITP patients

3.3 Platelet counts

The platelet count for each patient was the lowest for 6 months before application. The frequency distribution of platelet counts is shown in Fig. 3. The mean platelet count was 22.0 $\times$ 10^9/L in the total ITP patients (23.7 in females and 19.3 in males), 12.8 $\times$ 10^9/L in patients less than 15 years old, 27.2 $\times$ 10^9/L at 15–49 years old, and 21.1 $\times$ 10^9/L in patients more than 50 years old. Sixty-two percent of child patients had a platelet count of less than 10 $\times$ 10^9/L. This percentage is significantly higher ($p < 0.001$) than that (41.2%) in adult patients.

3.4 Hemorrhagic symptoms

The presenting clinical features are shown in Table 2. The most common presenting symptom was purpura (overall, 66.4%). The frequency of purpura (92.6%) and epistaxis (29.7%) in child ITP patients was significantly higher than that of purpura (62.8%) and epistaxis (10.0%) in adult patients. Cerebral vascular bleeding was noted as one of the presenting symptoms in 46 patients; one of these patients was a child, and the other 45 were adults.

3.5 Therapy

Therapies for child and adult ITP patients are summarized in Table 3. Therapies were significantly different between the child and adult patients. Two-thirds of child ITP patients received high-dose IgG therapy. In contrast, only 1,118 (16.3%) of adult ITP patients received such therapy. Prednisolone and the eradication of *Helicobacter pylori* in adult ITP patients were significantly higher (64.0 and 27.4%, respectively) compared with those in child ITP patients (44.0 and 1.9%, respectively). Approximately one-fifth of adult ITP patients received no medical therapy.

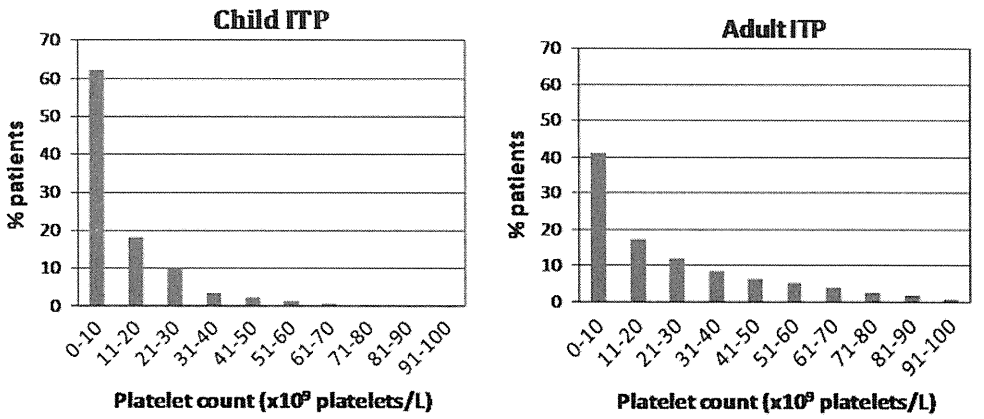


Fig. 3 Frequency distribution of the platelet counts in child and adult ITP patients

Table 2 Hemorrhagic symptoms of child and adult ITP

Bleeding symptoms	Overall (7,774 cases) Number of cases (%)	Child (929 cases) Number of cases (%)	Adult (6,845 cases) Number of cases (%)	*p* value (Child vs. adult)
Purpura	5,160 (66.4)	860 (92.6)	4,300 (62.8)	$p < 0.001$
Gingival bleeding	1,540 (19.8)	175 (18.8)	1,365 (19.9)	ns
Epistaxis	963 (12.4)	276 (29.7)	687 (10.0)	$p < 0.001$
Hematuria	507 (6.5)	54 (5.8)	453 (6.6)	ns
Melena	302 (3.9)	43 (4.6)	259 (3.8)	ns
Hypermenorrhea	275 (3.5)	11 (1.2)	264 (3.9)	$p < 0.001$
Cerebral bleeding	46 (0.6)	1 (0.1)	45 (0.7)	$p < 0.05$
Other bleeding	268 (3.4)	54 (5.8)	214 (3.1)	$p < 0.001$

ns not significant

Table 3 Therapy for child and adult ITP

Treatment	Overall (7,774 cases) Number of cases (%)	Child (929 cases) Number of cases (%)	Adult (6,845 cases) Number of cases (%)	*p* value (Child vs. adult)
Prednisolone	4,793 (61.7)	409 (44.0)	4,384 (64.0)	<0.001
Eradication of *H. pylori*	1,895 (24.4)	18 (1.9)	1,877 (27.4)	<0.001
High-dose IgG	1,731 (22.3)	613 (66.0)	1,118 (16.3)	<0.001
Splenectomy	233 (3.0)	5 (0.5)	228 (3.3)	<0.001
Immunosuppressant	172 (2.2)	6 (0.6)	166 (2.4)	<0.001
Danazol	117 (1.5)	4 (0.4)	113 (1.7)	<0.005
No therapy	1,643 (21.1)	129 (13.9)	1,514 (22.1)	<0.001

Table 4 Incidence of patients with ITP (All ages)

Study (year)	Number of patients	Design	Country	Annual population base	Inclusion criteria	Incidence (year studied)
Takahashi et al. [9]	523	Retrospective by registered data	Japan	ND	Plt. count $<100 \times 10^9$/L	2.28 (2001–2002)
Schoonen et al. [11]	1,145	Retrospective by administrative data	England	1.2–2.3 million	Plt. count: ND	3.9 (1990–2005)
This study (2010)	7,774	Retrospective by registered data	Japan	90.0 million	Plt. count $<100 \times 10^9$/L	2.16 (2004–2007)

ND not described; *plt. count* platelet count

4 Discussion

4.1 Incidence rate

We identified an annual incidence of 2.16 per 100,000 persons in all ages, as well as 1.91 in child and 2.20 in adult ITP patients. Tables 4, 5, 6 show the incidence reported in the literature [3–12]. Table 4 presents the incidence of ITP patients (all ages) [9, 11], and Tables 5 [3–5, 7, 10] and 6 [6, 8, 12] show the incidence of ITP in children and adults, respectively. In these reports, the number of ITP patients and/or size of the population was very small compared with our reports. Our report is, to date, the largest one.

There are several reasons why the incidence varies widely across studies. The first is the inclusion criteria used to define an ITP case, especially the cut-off point of the platelet count. Some investigators [8, 10] used less than 50×10^9/L or 30×10^9/L as inclusion criteria because these platelet counts are threshold points for clinically meaningful bleeding, and the others [6, 9] used 100×10^9/L or 150×10^9/L. It is expected that there would be a marked difference in the incidence depending on whether they use a low or high platelet count as inclusion criteria. A recently published report [14] from an International Working Group recommends the platelet count to be less than 100×10^9/L as the threshold for diagnosis. Therefore, we consider that it is necessary to report epidemiologic data using 100×10^9/L as a cut-off point for the platelet count in ITP.

The second reason is that different study designs have been utilized in these studies and there was a marked difference in the method regarding how to search the records of ITP patients. Terrell et al. [15] reported a critical review of published reports on the incidence of ITP. Some

Table 5 Incidence of child ITP patients

Study (year)	Number of patients	Design	Country	Annual population base	Inclusion criteria	Incidence (year studied)
Zaki et al. [3]	60	Retrospective by clinical criteria	Kuwait	0.125 million	Below 14 years Plt. count: ND	12.5 (1981–1986)
Lilleyman [4]	70	Prospective by clinical criteria	England	0.48 million	Below 14 years Plt. count: ND	4.8 (1980–1994)
Bolton-Maggs et al. [5]	427	Prospective by questionnaire	England	13 million	Below 15 years Plt. count: ND	3 (1995–1996)
Zeller et al. [7]	92	Prospective by registered data	Norway	0.86 million	Below 14 years Plt. count: ND	5.3 (1996–1997)
Zeller et al. [10]	506	Prospective by questionnaire	Nordic	4.6 million	Below 14 years Plt. count $<30 \times 10^9$/L	4.8 (1998–2000)
This study (2010)	929	Retrospective by registered data	Japan	12.2 million	Below 14 years Plt. count $<100 \times 10^9$/L	1.91 (2004–2007)

ND not described, *plt. count* platelet count

Table 6 Incidence of adult ITP patients

Study (year)	Number of patients	Design	Country	Annual population base	Inclusion criteria	Incidence (year studied)
Frederiksen et al. [6]	221	Retrospective by ICD code	Denmark	0.368 million	More than 15 years old Plt. count $<100 \times 10^9$/L	2.68 (1973–1995)
Neylon et al. [8]	245	Prospective by clinical criteria	England	3.08 million	More than 16 years old Plt. count $<50 \times 10^9$/L	1.6 (1993–1999)
Abrahamson et al. [12]	840	Retrospective by administrative data	England	1.55 million	More than 18 years old Plt. count: ND	3.9 (1992–2005)
This study (2010)	6,845	Retrospective by registered data	Japan	77.8 million	More than 15 years old Plt. count $<100 \times 10^9$/L	2.20 (2004–2007)

ND not described, *plt. count* platelet count

investigators analyzed the ITP patients seen in their hospital retrospectively [3] or in their region prospectively [4, 8]. These studies apparently include all newly diagnosed ITP patients. However, the numerator only included patients seen at one hospital or in one region. Other investigators sent questionnaires to pediatricians and hematologists in their regions to assess whether or not they had seen new patients presenting with ITP [5, 10]. The accuracy of active surveillance using a questionnaire depends on the response rate. A low response rate could have resulted in an underestimation of the incidence of ITP. However, we and other investigators [6, 7, 9, 11, 12] performed a prospective or retrospective cohort analysis of patients registered in the data bank. The diagnosis of ITP was based on administrative or discharge codes [11, 12] without a chart review to validate the diagnosis. Segal et al. [16] reported that estimation of the prevalence of ITP using the coding system was not so accurate, particularly when outpatient data were used. In our study, the initial diagnosis of ITP in the health care system for intractable diseases in Japan is rather accurate, since the hematological data including bone marrow examination are further validated by the Committee of the Department of Health and Medical Care of the regional prefecture.

Our study has some limitations as well. Firstly, our registration system is not mandatory. ITP patients themselves apply for recognition of the specified disease to the Department of Health and Medical Care of the regional prefecture according to their physician's advice. Some patients do not apply because they do not want to receive financial support or the disease status is mild. In this case, the incidence might be underestimated. Secondly, some possibilities exist that thrombocytopenic patients except those with ITP apply to obtain financial support. Aplastic anemia is already recognized as a specified disease in Japan. However, myelodysplastic syndrome is not recognized as a specified disease. Therefore, patients with myelodysplastic syndrome may apply for specified disease recognition. In this case, the incidence might be overestimated. The Committee of the Department of Health and

Medical Care of the regional prefecture check the hematological data, especially the differential count of peripheral white blood cells, and the results of bone marrow examination. They reject the application if myelodysplastic syndrome or other thrombocytopenic disorders cannot be excluded.

4.2 Age and sex distribution

Our data show a male preponderance among pediatric ITP patients among children aged 4 years or younger. These findings are in line with earlier reports [10, 17–19] of a male preponderance in early childhood ITP and male-to-female ratios decreasing from infancy to adolescence.

It has previously been reported that most cases of ITP occur between 20 and 40 years old, and that it is less common after 50 years old. We identified an increasing annual ITP incidence with age. The maximum age-specific incidence was in the eighth decade. This is in contradiction to the generally accepted epidemiological data [19, 20]. The observed findings regarding the ITP incidence by age in this study were similar to those of previous studies reported by Neylon et al. [8] and Frederiksen et al. [6], whereby both the groups estimated a higher incidence of ITP among those aged 60 years and older. These changes may reflect the changing age profile of the general population.

The incidence among both men and women began to increase after the age of 50. The incidence among women was higher than in men until 65, but not thereafter; there was no gender-specific relative differences in the age group of 65–74 years old. Schoonen et al. [11] also reported similar data. Of special interest is that gender-specific relative differences shifted to men with a higher incidence than women in the 75-year-old and older age groups. There are no reports that men show a higher incidence than women in the 75-year-old and older age groups.

4.3 Platelet counts and hemorrhagic symptoms

The platelet count in child ITP was very low compared with that in adult ITP (Fig. 3). Especially, the frequency of cases with a platelet count of 10,000 or less was high in child ITP. It is well-known that the platelet count is very low in patients with acute ITP. The platelet count in child ITP may be low since the major form of ITP in childhood is acute ITP [20].

The frequency of purpura and nasal bleeding in child ITP was significantly higher than in adult ITP. We considered that this also reflected the result that the acute form of ITP is frequent in childhood, as mentioned above [20]. On the other hand, the frequency of cerebral bleeding in patients with adult ITP was very high. This suggests that cases of cerebral bleeding might increase with the advancing age of patients with ITP.

4.4 Therapy

A variety of therapies were performed for ITP patients. Patients who received no treatment comprised 1.39% of those with child ITP, and 22.1% of those with adult ITP. The most frequently performed therapy for child ITP was high-dose IgG therapy, and 66.0% of child patients received this therapy. Several explanations may be considered: (1) the dosage of IgG in childhood may be low, so the cost would be cheaper than for adult ITP, (2) acute ITP is the major form in childhood, and this form involves serious hemorrhagic symptoms [20], so a rapid rise in the platelet count may be needed. Recently, in Japan, therapy involving the eradication of *Helicobacter pylori* has become widespread, and this study revealed that 27.4% of adult ITP patients received this therapy. There is convincing evidence [21] that the eradication of *Helicobacter pylori* is very effective to treat adult ITP patients, especially in Japan and Italy.

In summary, our report provides a population-based estimate of the incidence of ITP in Japan. The overall incidence was 2.16 per 10^5 per annum. This study is the largest reported to date. This population-based study suggests that the incidence of ITP increases with age, showing a biphasic distribution, with most patients presenting during the seventh and eighth decades, suggesting that the traditional view of adult ITP as being a disease that affects predominantly young females needs to be modified.

Acknowledgments This study was supported in part by a Grant-in Aid for Blood Coagulation Abnormalities from the Ministry of Health, Labour, and Welfare of Japan.

References

1. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. Blood. 2009;113: 6511–21.
2. McMillan R, Wang L, Tomer A, Nichol J, Pistillo J. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. Blood. 2004;103: 1364–9.
3. Zaki M, Hassanein AA, Khalil AF. Childhood idiopathic thrombocytopenic purpura: report of 60 cases from Kuwait. J Trop Pediatr. 1990;36:10–3.
4. Lilleyman JS. Intracranial haemorrhage in idiopathic thrombocytopenic purpura. Arch Dis Child. 1994;71:251–3.
5. Bolton-Maggs PH, Moon I. Assessment of UK practice for management of acute childhood idiopathic thrombocytopenic purpura against published guidelines. Lancet. 1997;350:620–3.
6. Frederiksen H, Schmidt K. The incidence of idiopathic thrombocytopenic purpura in adults increases with age. Blood. 1999;94:909–13.

7. Zeller B, Helgestad J, Hellebostad M, Kolmannskog S, Nystad T, Stensvold K, Wesenberg F. Immune thrombocytopenic purpura in childhood in Norway: a prospective, population-based registration. Pediatr Hematol Oncol. 2000;17:551–8.
8. Neylon AJ, Saunders PWG, Howard MR, Proctor SJ, Taylor PRA. Clinically significant newly presenting autoimmune thrombocytopenic purpura in adults: a prospective study of a population-based cohort of 245 patients. Br J Haematol. 2003;122:966–74.
9. Takahashi I, Yorimitsu S. Epidemiological aspects of idiopathic thrombocytopenic purpura in Kochi prefecture. Jpn J Clin Hematol. 2004;45:372–7. (in Japanese).
10. Zeller B, Rajantie J, Hedlund-Treutiger I, Tedgård U, Wesenberg F, Jonsson OG, Henter JI. NOPHO ITP. Childhood idiopathic thrombocytopenic purpura in the Nordic countries: epidemiology and predictors of chronic disease. Acta Paediatr. 2005;94:178–84.
11. Schoonen WM, Kucera G, Coalson J, Li L, Rutstein M, Mowat F, et al. Epidemiology of immune thrombocytopenic purpura in the general practice research database. Br J Haematol. 2009;145:235–44.
12. Abrahamson PE, Hall SA, Feudjo-Tepie M, Mitrani-Gold FS, Logie J. The incidence of idiopathic thrombocytopenic purpura among adults: a population-based study and literature review. Eur J Haematol. 2009;83:83–9.
13. George JN, Woolf SH, Raskob GE, Wasser JS, Aledort LM, Ballem PJ, et al. Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. Blood. 1996;88:3–40.
14. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnord DM, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009;113:2386–93.
15. Terrell DR, Beebe LA, Vesely SK, Neas BR, Segal JB, George JN. The incidence of immune thrombocytopenic purpura in children and adults: a critical review of published reports. Am J Hematol. 2010;85:174–80.
16. Segal JB, Powe NR. Accuracy of identification of patients with immune thrombocytopenic purpura through administrative records: a data validation study. Am J Hematol. 2004;75:12–7.
17. Sutor AH, Harms A, Kaufmehl K. Acute immune thrombocytopenia (ITP) in childhood: retrospective and prospective survey in Germany. Sem Thromb Hemost. 2001;27:253–67.
18. Yong M, Schoonen WM, Li L, Kanas G, Coalson J, Mowat F, Fryzek J, Kaye JA. Epidemiology of paediatric immune thrombocytopenia in the General Practice Research Database. Br J Haematol. 2010;149:855–64.
19. Doan CA, et al. Idiopathic and secondary thrombocytopenic purpura: clinical study and evaluation of 381 cases over a period of 28 years. Ann Intern Med. 1960;53:861–76.
20. George JN, El-Harake MA, Aster RH. Thrombocytopenia due to enhanced platelet destruction by immunologic mechanisms. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, editors. Williams hematology. New York: McGraw-Hill; 1995. p. 1315–55.
21. Fujimura K, Kuwana M, Kurata Y, Imamura M, Harada H, Sakamaki H, et al. Is eradication therapy useful as the first line of treatment in Helicobacter pylori-positive idiopathic thrombocytopenic purpura? Analysis of 207 eradicated chronic ITP cases in Japan. Int J Hematol. 2005;81:162–8.

Journal of Thrombosis and Haemostasis, 9: 1040–1048 DOI: 10.1111/j.1538-7836.2011.04245.x

ORIGINAL ARTICLE

Bleeding tendency and impaired platelet function in a patient carrying a heterozygous mutation in the thromboxane A_2 receptor

T. KAMAE,* K. KIYOMIZU,* T. NAKAZAWA,* S. TADOKORO,* H. KASHIWAGI,* S. HONDA,† Y. KANAKURA* and Y. TOMIYAMA*‡

Department of Hematology and Oncology, Osaka University Graduate School of Medicine; †National Cardiovascular Center Research Institute; and ‡Department of Blood Transfusion, Osaka University Hospital, Suita, Osaka, Japan

To cite this article: Kamae T, Kiyomizu K, Nakazawa T, Tadokoro S, Kashiwagi H, Honda S, Kanakura Y, Tomiyama Y. Bleeding tendency and impaired platelet function in a patient carrying a heterozygous mutation in the thromboxane A_2 receptor. *J Thromb Haemost* 2011; **9**: 1040–8.

Summary. *Background:* Thromboxane A_2 receptor (TXA_2R) abnormality appears to dominantly disturb platelet function. *Objectives*: To reveal a molecular genetic defect in a patient with TXA_2R abnormality and investigate the mechanism for the impaired response to TXA_2. *Patient*: The proband (OSP-2, PT) was a 7-year-old Japanese girl, suffering from repeated mucocutaneous bleeding. *Methods and results*: U46619 (2.5 and 10 μM)-induced platelet aggregation was remarkably impaired in the proband and her father. Immunoblots showed that TXA_2R expression levels in their platelets were approximately 50% of controls, and nucleotide sequence analysis revealed that they were heterozygous for a novel mutation, c.167dupG in the TXA_2R cDNA. Expression studies using Chinese hamster ovary (CHO) cells indicated that the mutation is responsible for the expression defect in TXA_2R. We then examined $\alpha_{IIb}\beta_3$ activation by employing an initial velocity analysis and revealed that U46619 failed to induce a sustained $\alpha_{IIb}\beta_3$ and Rap1B activation in the proband. In addition, platelet secretion as monitored by P-selectin expression was markedly impaired in response to U46619 but not to ADP. The interaction between secreted ADP and $P2Y_{12}$ has been shown to play a critical role in the sustained $\alpha_{IIb}\beta_3$ activation (Kamae *et al. J Thromb Haemost* 2006; **4**: 1379). As expected, small amounts of exogenous ADP (0.5 μM) partially restored the sustained $\alpha_{IIb}\beta_3$ activation induced by U46619. *Conclusion:* Our present data strongly suggest that the impaired platelet activation in response to U46619 in the heterozygous subject for the TXA_2R mutation is, at least in part, as a result of the decrease in ADP secretion.

Keywords: $\alpha_{IIb}\beta_3$, GPCR, $P2Y_{12}$, Rap1B, TXA_2 receptor.

Correspondence: Yoshiaki Tomiyama, Department of Blood Transfusion, Osaka University Hospital, 2-15 Yamadaoka, Suita, Osaka 565-0871, Japan.
Tel.: +81 6 6879 5887; fax: +81 6 6879 5889.
E-mail: yoshi@hp-blood.med.osaka-u.ac.jp

Received 14 January 2011, accepted 13 February 2011

Introduction

Platelets play a critical role in hemostasis and thrombosis. At sites of vascular injury, platelets adhere to altered vascular surfaces or exposed subendothelial matrices, then become activated and aggregate each other. Activated platelets bring about amplification of activation signals by producing and/or secreting platelet agonists by themselves and the recruitment of other platelets at the site of vascular injury [1,2]. Thromboxane A_2 (TXA_2), an arachidonic acid metabolite, and ADP, one of the dense granule contents, are major platelet agonists that amplify platelet activation *in vivo*. It is well known that the $P2Y_{12}$ ADP receptor inhibitor, as well as aspirin, an inhibitor of TXA_2 synthesis, decreases thrombotic events such as myocardial infarction and brain stoke, suggesting that these agonists are important for hemostasis and thrombosis [3–5].

Characterization of inherited platelet function disorders has also revealed critical molecules and receptors for hemostasis and thrombosis [6,7]. TXA_2 receptor (TXA_2R) is a G protein-coupled receptor (GPCR), encoded by the *TBXA2R* gene [8]. TXA_2R exists as two alternatively spliced isoforms, α and β, which differ only in their C-terminus. Platelets predominantly express $TXA_2R\alpha$, while both isoforms are expressed in vascular tissues such as endothelial cells and vascular smooth muscle cells [9,10]. To date, only two molecular genetic abnormalities in TXA_2R which are associated with a mild bleeding tendency have been reported: R60L and D304N [11–13]. The R60L and D304N substitutions caused normal receptor expression but the variant receptors were dysfunctional. In general, platelet receptor abnormalities such as Glanzmann thrombasthenia and $P2Y_{12}$ deficiency are inherited in an autosomal recessive manner. In other words, a subject carrying a heterozygous mutation does not show any platelet functional abnormality. However, previously identified heterozygous mutations in TXA_2R both showed platelet functional abnormality [11,13], and its mechanism still remains to be clarified.

In the present study, we examined a patient with a bleeding tendency and platelet dysfunction, and demonstrated that the

patient is heterozygous for a novel mutation in TXA_2R, c.167dupG, which is responsible for the expression defect in the receptor. In addition, we carefully examined the effect of the heterozygous mutation on platelet function.

Materials and methods

This study has been approved by the research ethics committee of Osaka University. Blood samples were obtained with written informed consent in accordance with the Declaration of Helsinki.

Reagents

ADP, protease-activated receptor 1-activating peptide (PAR1 TRAP, SFLLRNPNDKYEPF), thrombin, TXA_2 analog U46619 (9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin $F_{2\alpha}$) and prostaglandin E_1 (PGE_1) were purchased from Sigma-Aldrich (St Louis, MO, USA). FITC-PAC-1, a ligand-mimetic $\alpha_{IIb}\beta_3$-specific monoclonal antibody (mAb) that binds specifically to activated $\alpha_{IIb}\beta_3$, was bought from Becton Dickinson Biosciences (Mountain View, CA, USA). PE-labeled anti-CD62p (P-selectin) and PE-IgG were purchased from Beckman Coulter (Brea, CA, USA). A specific TXA_2R antagonist, SQ-29548, was purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-human TXA_2R polyclonal antibody which recognizes C-terminal amino acids 323–343 (LSTRPRSLSLQPQLTQRSGLQ) of the receptor was purchased from Cayman Chemical. Anti-β-actin antibody and monoclonal anti-FLAG (M2) antibody were purchased from Sigma-Aldrich.

Platelet preparation

Platelet-rich plasma (PRP) was prepared as previously described [14]. In brief, fresh whole blood samples anticoagulated with 0.1 volume of 0.38% sodium citrate were obtained from the proband, her family members and healthy volunteers, who had not taken any medication for at least 1 week. The samples were centrifuged at 250×*g* for 10 min and allowed to rest for 30 min before use.

Washed human platelets were prepared as previously described [14]. In brief, the PRP after incubation with 20 ng mL^{-1} PGE_1 for 15 min was centrifuged at 750×*g* for 10 min, washed with 0.05 M isotonic citrate buffer containing 20 ng mL^{-1} PGE_1, resuspended in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3.3 mM NaH_2PO_4, 3.8 mM HEPES, 0.1% glucose, 0.1% bovine serum albumin, pH 7.4) without PGE_1 and allowed to rest for 30 min before use.

Platelet aggregation studies

Platelet aggregation to various agonists was measured in PRP using aggregometer MCM hema tracer 313 M as previously described [15]. The aggregation responses were compared with responses with normal control PRP collected at the same time as the proband and her family members.

Nucleotide sequence analysis of TXA_2R

Total cellular RNA was separated from platelets of the proband, her family members and normal controls using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). TXA_2R mRNA was specifically amplified by RT-PCR using Omniscript RT Kits (Qiagen, Velno, The Netherlands) [16]. The primers, 1s and 8r, were constructed based on the published sequence of TXA_2R cDNA and used for the first round PCR for TXA_2R cDNA. The sequencing reaction was performed with primers described below using a BIGDYE TERMINATOR V3.1 cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Subcloning of TXA_2R cDNA was performed using the T/A cloning technique (Mighty T/A cloning kit; Takara, Tokyo, Japan).

Genomic DNA was prepared from mononuclear cells of the proband, her family members and healthy volunteers using a DNA extraction kit (SepaGene; Sanko Junyaku, Tokyo, Japan), and the TXA_2R coding sequences were analyzed. Primers used in this study are described in the supplemental Table S1.

Immunoblot analysis of TXA_2R

The levels of platelet TXA_2R in the proband, her family members and normal controls were studied by immunoblot analysis. In all, 20×10^6 washed platelets were lysed with RIPA buffer (25 mM Tris-HCl pH7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail) and added to 2× sample buffer contained 5% β-mercaptoethanol (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue). Next, 20 μg proteins was separated using 5–20% gradient gel using a mini-gel electophoresis system (Bio-Rad, Hercules, CA, USA). After transfer to polyvinylidene difluoride (PVDF) membranes, TXA_2R was detected with a rabbit anti-TXA_2R polyclonal antibody (Cayman Chemical). To confirm that each lane contained the same amounts of platelet proteins, the total beta-actin in each lysate was detected with anti-β-actin antibody in parallel. The optical density of the bands was measured using IMAGE-J software (Bethesda, MD, USA). The amount of TXA_2R was compared with the average of five healthy volunteer donors.

Surface expression of the platelet glycoproteins

Surface expression levels of platelet glycoproteins were measured with various monoclonal antibodies (CD41, CD36, CD42b, CD61, GPVI and GPIa) (Becton Dickinson Biosciences) using flow cytometry (FACSCalibur, Becton Dickinson) as described previously [16].

Ligand binding studies and expression of CD62p (P-selectin)

For conventional binding assay, washed platelets adjusted to 50×10^3 μL^{-1} were stimulated with various agonists (0.2 U mL^{-1} thrombin, 5 μM U46619, 20 μM ADP) under static conditions and incubated with FITC-PAC-1 and PE-anti-CD62p for 30 min and analyzed using flow cytometry [14].

To evaluate more precisely the dynamic changes in the $\alpha_{IIb}\beta_3$ activation state, we performed velocity analysis for PAC-1 binding that has recently been developed [17,18]. In brief, FITC-PAC-1 and PE-anti-CD62p were added to the activated platelets (200×10^3 μL^{-1}) at the indicated time points (5 s, 1 min and 5 min) after stimulation and incubated for only 30 s to obtain the PAC-1 binding velocity at the time points in question. The velocity of PAC-1 binding and PE-anti-CD62p binding reflects the relative numbers of activated $\alpha_{IIb}\beta_3$ and expression of CD62p, respectively, at those time points.

Rap 1B activation assay

The detection of activated Rap 1B was performed using a pull-down assay kit according to the manufacturer's instructions (EZ-Detect™ Rap1 Activation Kit: Pierce, Rockford, IL, USA) as previously described [14]. In brief, platelets were stimulated with 5 μM U46619 for 5 s, 1 min and 5 min or 50 μM PAR1 for 1 min, and then lysed with 0.5% Triton-X100 lysis buffer. The guanosine triphosphate (GTP)-form of Rap 1B was pulled down by incubation with glutathione-S-transferase (GST)-RalGDS-Rap 1-binding domain (RBD) and glutathione beads for 1 h at 4 °C. After washing with lysis buffer, proteins were eluted from the precipitates with SDS-sample buffer with 2-mercaptoethanol at 100 °C for 5 min, and resolved by electrophoresis on a 5–20% SDS-PAGE gel. After transfer to PVDF membranes, Rap 1B was detected with rabbit anti-Rap 1 polyclonal antibody. The total Rap 1B in each lysate was detected in samples assayed in parallel. The optical density of the bands was measured using IMAGE-J software.

TXA_2R expression experiments

Wild-type (WT) TXA_2R cDNA fused at the N-termimus with the FLAG epitope tag was made from control platelet cDNA using a PCR technique and inserted into the multi-cloning site of the expression vector pcDNA3.1. The mutant (c.167dupG) expression construct was made by site-directed mutagenesis using the QuickChange site-directed Mutagenesis Kit (Stratagene, San Diego, CA, USA) according to the manufacturer's instructions. WT or the mutant TXA_2R expression construct was co-transfected with the pDsRed2-N1 vector which encodes red fluorescent protein DsRed2 using lipofectamin 2000 into Chinese hamster ovary (CHO) cells [18]. Expression of the FLAG-tagged TXA_2R in transfected cells was detected with the anti-FLAG M2 monoclonal antibody and FITC-anti-mouse IgG (Sigma) using flow cytometry. Propidium iodide staining was used for exclusion of dead cells.

Results

Case study

The proband (OSP-2, PT) was a 7-year-old Japanese girl who is the second daughter from non-consanguineous parents. The proband had been suffering from cutaneous ecchymosis and recurrent epistaxis from 3 years old. She also suffered from allergic rhinitis. Occasionally the epistaxis lasted over an hour and local measures were necessary to stop the epistaxis. There was no family history of a bleeding tendency. At Hyogo Medical Collage, a slightly prolonged bleeding time (7.5 min, normal < 5 min by Duke's method) and an impaired platelet aggregation induced by ADP was observed. To further examine her bleeding tendency, the proband was referred to Osaka University Hospital in September 2008. The proband showed a normal platelet count (212×10^3 μL^{-1}) and normal coagulation tests (prothrombin time, activated partial thromboplastin time and D-dimer). Coagulation factors (Fibrinogen, VIII, IX and XIII) and von Willebrand factor activity (66%) were also within the normal range.

Platelet aggregation studies

We first examined the expression of platelet membrane glycoproteins in the proband using flow cytometry. The proband's platelets normally express GPIb-IX-V, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), $\alpha_2\beta_1$, GPVI and CD36 (data not shown). Figure 1 shows platelet aggregation using PRP obtained from

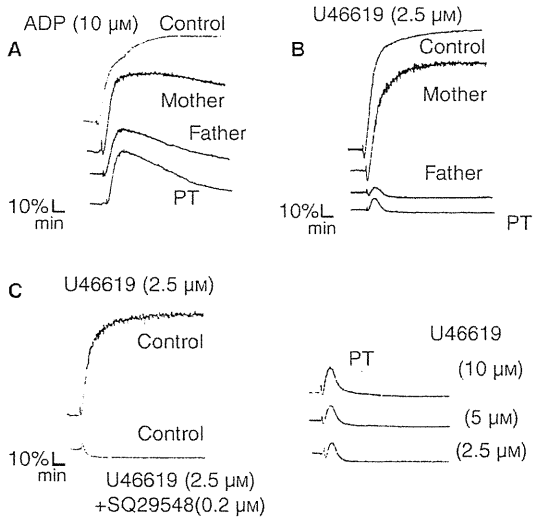


Fig. 1. Agonist induced platelet aggregation in the proband (PT) and her family members. (A, B) Platelets aggregation studies were performed using platelet-rich plasma (PRP) obtained from PT, her father, her mother and normal controls. PRP was stimulated with 10 μM ADP or 2.5 μM U46619. (C) Left: PRP obtained from normal controls was stimulated with 2.5 μM U46619, and U46619 plus 0.2 μM SQ-29548, a thromboxane A_2 receptor (TXA_2R) antagonist. Right: PRP obtained from PT was stimulated with various concentrations of U46619 (up to 10 μM). These results are representative of at least two experiments.

the proband (PT), her father or her mother in response to various agonists. Under our experimental conditions, every healthy control subject showed full platelet aggregation with 10 μM ADP stimulation. However, 10 μM ADP-induced platelet aggregation in her father as well as the proband was impaired with only a reversible aggregation, suggesting a defect in ADP-induced secondary aggregation. Moreover, the platelet aggregation in her father as well as the proband was markedly impaired with only a small and transient aggregation in response to 2.5 μM U46619, a TXA_2 analog, and the aggregation was still markedly impaired even at a high concentration of U46619 (10 μM). As SQ-29548, a TXA_2 receptor antagonist, totally abolished platelet aggregation induced by 2.5 μM U46619 in a control subject, the presence of the transient aggregation suggested that TXA_2-mediated signaling was not totally absent but partially existed in her father as well as the proband. Her mother's platelets aggregated normally in response to these agonists. Platelets obtained from her two sisters or one elder brother aggregated normally in response to 2.5 μM U46619 (data not shown).

Expression levels of platelet TXA_2R

Using an immunoblot assay, we then examined the amount of TXA_2 receptor in platelets obtained from the proband, her family members, or five healthy controls using polyclonal antibody recognizing C-terminal amino acids 323–343 (LSTRPRSLSLQPQLTQRSGLQ) of the receptor. Various amounts of platelet proteins obtained from controls were analyzed in parallel to obtain a standard curve. As shown in Fig. 2, the amounts of TXA_2R were 45.5 ± 5.4% and 52.7 ± 14.4% of controls for the proband and her father, respectively (n = 3). An abnormal TXA_2R with different molecular weights was not detected in the proband or her father.

Sequence analysis of TXA_2R cDNA and genomic DNA

In order to reveal the molecular defect in the proband, TXA_2R cDNAs which cover the entire coding region of TXA_2R were amplified by RT-PCR from the proband and control platelet mRNA and sequenced. As shown in Fig. 3, a novel mutation (duplication at c.167, c.167dupG) was detected in the proband. Subcloning of the proband's platelet cDNA and genomic DNA with the T/A cloning technique confirmed that the proband was heterozygous for the mutation (data not shown). The same mutation was detected in her father's platelet cDNA, indicating that the mutant allele was derived from her father. No other mutations were detected in the entire coding region of TXA_2R in either the proband or her father. There was no abnormality in the coding region of TXA_2R in her mother.

The c.167dupG would lead to a frame shift from amino acid 58 and a late termination at amino acid 389. Thus, the mutant protein, if expressed, would have different amino acids 58–389 from the wild-type TXA_2R. To examine if the mutant TXA_2R would be expressed, wild-type or mutant TXA_2R expression construct in pcDNA3.1 was co-transfected with the pDsRed2-N1 vector into CHO cells. In this over expression assay, the expression levels of the mutant TXA_2R in the dsRED positive CHO cells were remarkably impaired compared with the

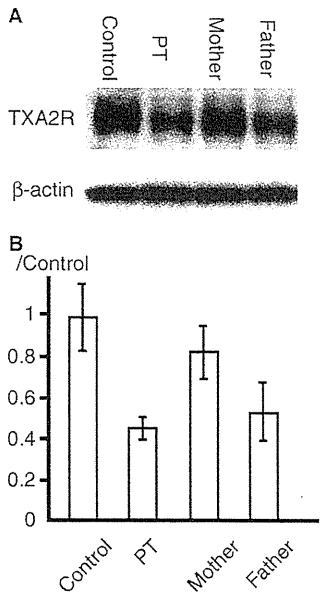


Fig. 2. Expression levels of platelet thromboxane A_2 receptor (TXA_2R). (A) In all, 20 μg platelet proteins obtained from the proband (PT), her father, her mother and healthy controls were separated using 5–20% gradient gel, and transferred to polyvinylidene difluoride membranes. TXA_2R was detected by immunoblot assay with rabbit anti-TXA_2R polyclonal antibody which recognizes C-terminal amino acids. The total β-actin in each lysate was detected with anti-β-actin antibody in parallel. Representative data are shown. (B) The optical density of the bands was measured using IMAGE-J software. Three experiments were quantified and were expressed as a relative ratio compared with the average of five healthy controls. Error bar represents the mean ± SD of three experiments.

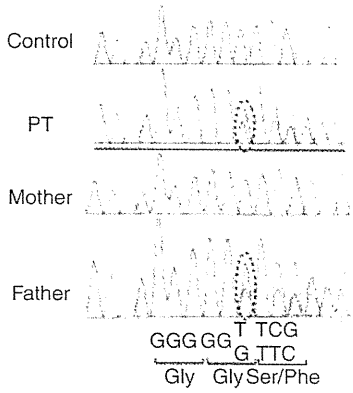


Fig. 3. Nucleotide sequence analysis of thromboxane A_2 receptor (TXA_2R) cDNA. TXA_2R cDNAs covering the entire coding region of TXA_2R were amplified from the proband (PT), her father, her mother and control platelet mRNA by RT-PCR and sequenced. c.167dupG was detected heterozygously in the PT and her father's cDNA.

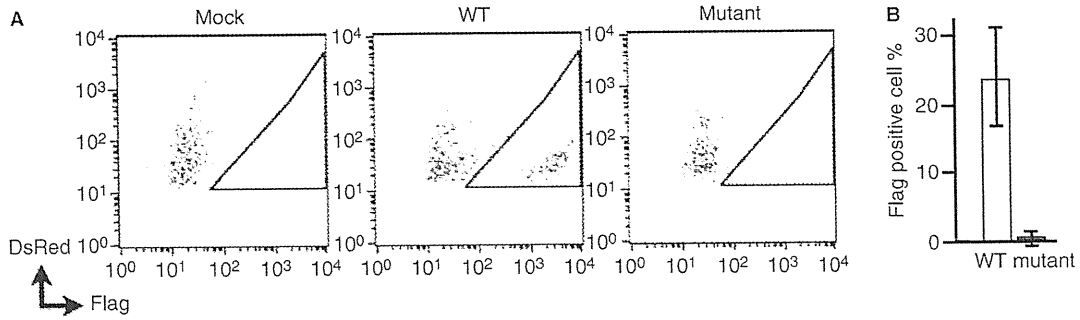


Fig. 4. Surface expression of recombinant wild-type (WT) and the mutant thromboxane A_2 receptor (TXA_2R) on CHO cells. FLAG-tagged wild-type (WT) or mutant TXA_2R expression construct in pcDNA3.1 was co-transfected with pDsRed2-N1 vector into Chinese hamster ovary (CHO) cells. DsRed-positive cells were gated, and then surface expression of the FLAG-tagged WT or mutant TXA_2R was detected with an anti-FLAG M2 monoclonal antibody using flow cytometry. (A) A representative experiment is shown. (B) Percentage of FLAG positive cells (gated area) in the DsRed positive cell population is shown for WT and the mutant TXA_2R. Error bar represents mean ± SD of three experiments.

expression levels in the WT TXA_2R (4.5% ± 2.2 of the WT, n = 3) (Fig. 4). These data indicate that the mutation leads to a markedly impaired expression of the TXA_2R. Taken together with the immunoblot assay, it is likely that approximately 50% of normal TXA_2R may exist in platelets from the proband and her father who are heterozygous for the mutation.

Both $\alpha_{IIb}\beta_3$ activation and P-selectin expression were impaired on platelets from her father as well as the proband in response to U46619 stimulation

We then examined $\alpha_{IIb}\beta_3$ activation employing PAC-1 mAb with a conventional binding assay. In brief, washed platelets were incubated with different kinds of agonists in the presence of FITC-PAC-1 and PE-anti-CD62p for 30 min and then analyzed using flow cytometry. Normal PAC-1 binding and P-selectin expression were induced with the stimulation of 0.5 U mL^{-1} thrombin or 20 μM ADP on the proband's platelets, while both PAC-1 binding and P-selectin expression were markedly impaired with 5 μM U46619 stimulation (Fig. 5). Essentially the same defects were obtained in her father's platelets. The mean fluorescence intensities (MFI) for FITC-PAC-1 binding with 5 μM U46619 stimulation were 101.8 ± 35.9, 5.7 ± 1.9 and 6.0 ± 1.4 (mean ± SD, n = 4) for controls, the proband and her father, respectively. The MFIs for PE-CD62p were 76.6 ± 38.1, 8.4 ± 6.1 and 10.2 ± 6.9 (mean ± SD, n = 4), for controls, the proband and her father, respectively.

Initial velocity assay reveals transient but substantial $\alpha_{IIb}\beta_3$ activation on the proband's platelets with 5 μM U46619 stimulation

To evaluate more precisely the dynamic changes in the $\alpha_{IIb}\beta_3$ activation, we performed initial velocity analysis for PAC-1 binding. In brief, FITC-PAC-1 and PE-anti-CD62p were added to the activated platelets at the indicated time points (5 s, 1 min or 5 min) after stimulation and incubated for only 30 s [17,18]. We detected PAC-1 binding about 36–42% of control on platelets from the proband and her father at 5 s after stimulation with 5 μM U46619. However, at 1 min and 5 min PAC-1 binding velocity in the proband and her father rapidly decreased, suggesting that sustained $\alpha_{IIb}\beta_3$ activation rather than initiation of $\alpha_{IIb}\beta_3$ activation was impaired. In contrast, the initial velocity assay showed that P-selectin expression was impaired at any time points examined (Fig. 6). Thus, our initial velocity assay suggests that transient but substantial $\alpha_{IIb}\beta_3$ activation occurred with impaired granule release reaction on platelets from the proband and her father.

Transient but substantial Rap 1B activation in the late phase of the father's platelets stimulated with U46619

We next examined the dynamic changes of Rap1B activation, as Rap1B has been demonstrated to be a regulator of $\alpha_{IIb}\beta_3$ activation in platelets. The proband and her father showed essentially the same defects in platelet function as described above. Therefore, we used the father's platelets hereafter because of easy accessibility of the samples. As Rap 1B activation induced by 50 μM PAR1 in the father was similar to control platelets, Rap 1B activation induced by 5 μM U46619 was compared with that by 50 μM PAR1 and expressed as a ratio. As shown in Fig. 7, the ratios of Rap1B activation in control platelets were 1.00 ± 0.19, 1.18 ± 0.11, 0.87 ± 0.08, respectively (mean ± SD, n = 3). In sharp contrast, those in the father's platelets were 0.50 ± 0.23, 0.17 ± 0.10, 0.11 ± 0.03 in 10 s, 1, and 5 min, respectively. Thus, Rap1B was substantially activated in the father's platelets with U46619 and the activation appeared to correlate with the transient $\alpha_{IIb}\beta_3$ activation (compare Figs 6 and 7).

Exogenous 0.5 μM ADP-induced sustained $\alpha_{IIb}\beta_3$ activation on the father's platelets stimulated with U46619

We have already demonstrated that the interaction of ADP and $P2Y_{12}$ is critical for sustained $\alpha_{IIb}\beta_3$ activation [14]. We, therefore, added exogenous 0.5 μM ADP to U46619-stimulated platelets. We confirmed that platelet aggregation was not induced at 0.5 μM ADP (data not shown). This concentration of ADP (0.5 μM) augmented $\alpha_{IIb}\beta_3$ activation in the initial

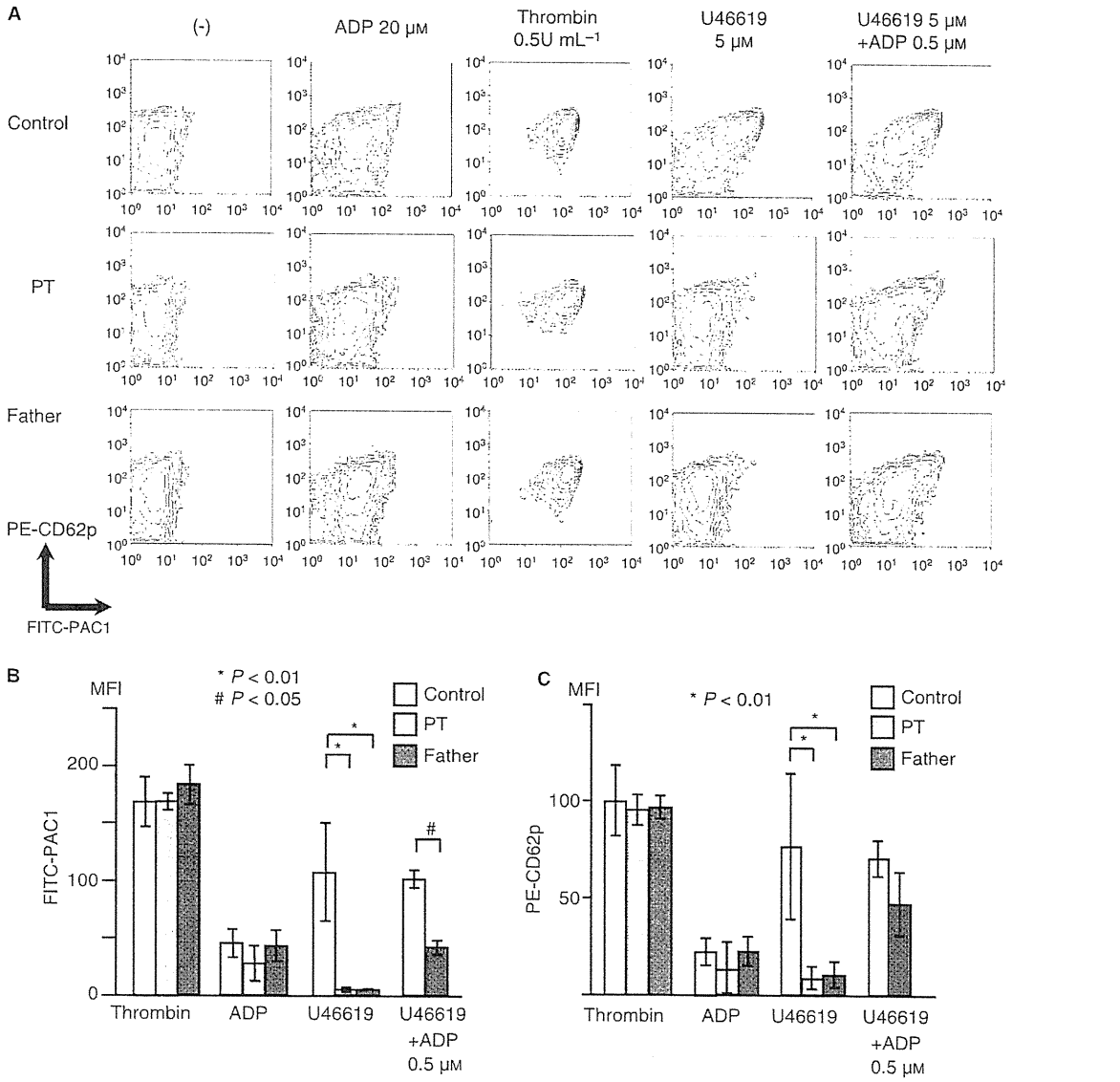


Fig. 5. Analysis of agonist-induced $\alpha_{IIb}\beta_3$ activation and P-selectin expression on platelet using a conventional binding assay. (A) Washed platelets (50×10^3 μL^{-1}) obtained from the proband (PT), her father and a normal control stimulated with 20 μM ADP, 0.5 U mL^{-1} thrombin, 5 μM U46619, or 5 μM U46619 plus 0.5 μM ADP were incubated with FITC-PAC1 and PE-CD62p for 30 min and analyzed using flow cytometry. (B, C) Mean MFIs for specific PAC-1 binding (B) or specific CD62p binding (C) to control (white bar), PT (gray bar), and her father's (dark gray bar) platelets stimulated with various agonists are shown. Error bar represents mean ± SD of three independent experiments.

velocity assay as well as in the conventional assay (Figs 5 and 6). The MFI of PAC-1 binding to the father's platelets stimulated with 5 μM U46619 increased to 43.3 ± 5.4 (mean ± SD n = 4) by 0.5 μM ADP (Fig. 5). The initial velocity assay clearly showed that $\alpha_{IIb}\beta_3$ activation was sustained even at 5 min after stimulation by the addition of 0.5 μM ADP (Fig. 6). Exogenous 0.5 μM ADP augmented P-selectin expression as well.

Discussion

In the present study, we have demonstrated a novel mutation (c.167dupG) in the *TBXA2R* gene leading to a frame shift and late termination of platelet TXA_2R associated with the expression defect in the receptor. The proband and her father were heterozygous for the mutation in the *TBXA2R* gene and expression levels of the TXA_2R on their platelets were reduced to 50% of controls. Expression studies using CHO cells confirmed that the mutation is responsible for the expression defect in the TXA_2R. Previously reported naturally occurring mutations in the TXA_2R are only missense mutations (R60L and D304N) which induce qualitative defects in the receptor [11–13]. Thus, the mutation reported in the present study is the first to induce the expression defect in TXA_2R.

Platelets from the proband and her father still expressed 50% of normal TXA_2R. Nevertheless, their platelets showed a

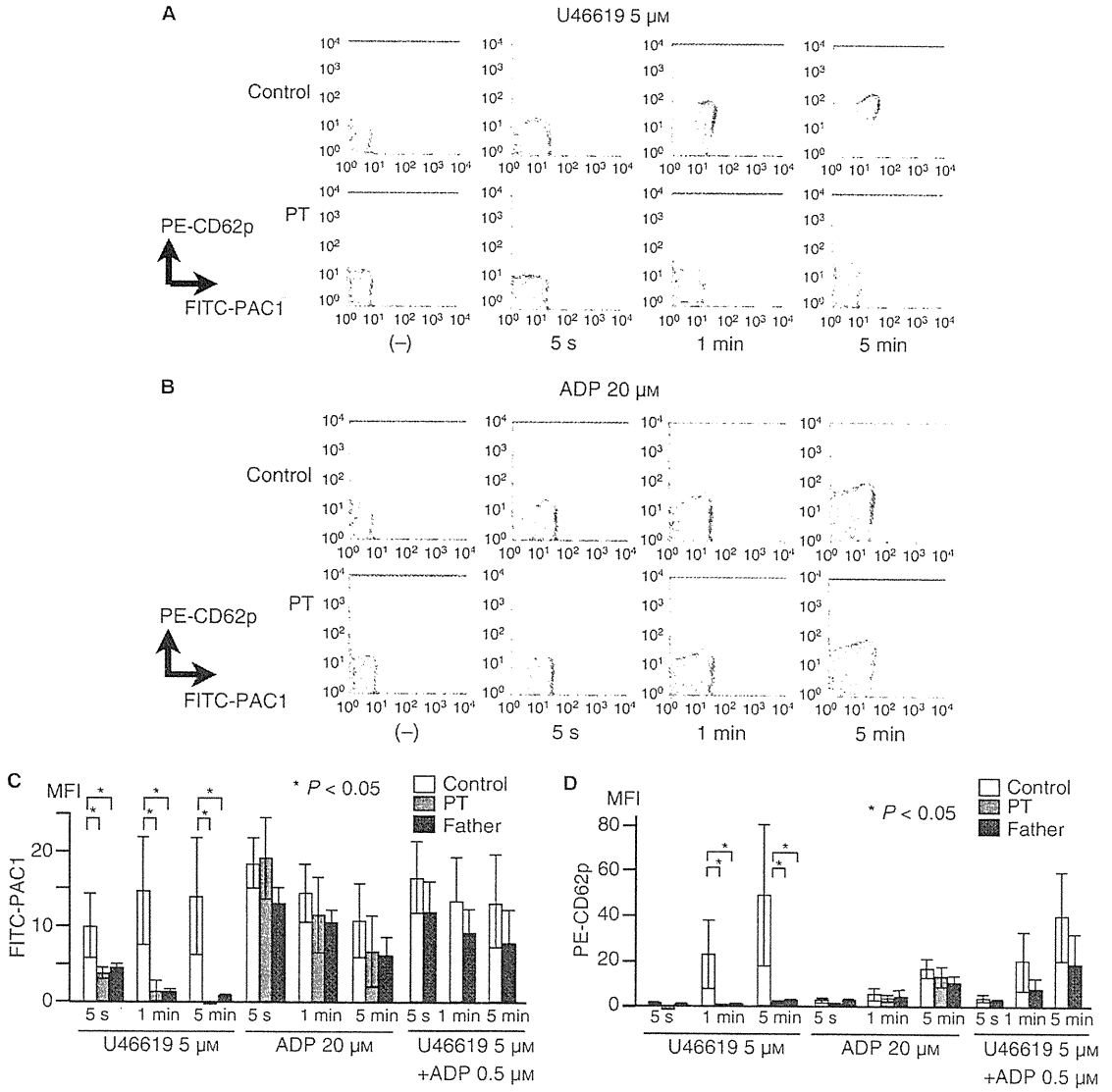


Fig. 6. Analysis of agonist-induced $\alpha_{IIb}\beta_3$ activation and P-selectin expression using an initial velocity binding assay. (A, B) PRP (200×10^3 μL^{-1}) was stimulated with 5 μM U46619 (A) or 20 μM ADP (B) for 5 s, 1 min and 5 min, and then FITC-PAC1 and PE-CD62p were added and incubated only for 30 s. Then, the binding of these antibodies was analyzed using flow cytometry. (C, D) Mean MFIs for specific PAC-1 binding (C) or specific CD62p binding (D) to the control (white bar), the proband (PT) (gray bar) and her father's (dark gray bar) platelets are shown. Error bar represents mean ± SD of three independent experiments.

markedly impaired response to U46619 even at a high concentration. Consistent with this abnormality, ADP-induced secondary aggregation was impaired, whereas thrombin and PAR1-induced platelet activation was normal (Figs 5 and 7). Thus, functional defects in the proband platelets reside exclusively in TXA_2R-mediated platelet activation. The R60L substitution in the intracellular loop 1 of the TXA_2R found in five unrelated Japanese patients with impaired TXA_2-induced platelet aggregation induced loss of TXA_2R function by abrogating Gq coupling to the receptor without disturbing ligand-binding capacity [11,12]. As compared with the proband (T.T.), his daughter who was heterozygous for the R60L mutation showed a similar but slightly milder functional defect in response to STA_2 (synthetic TXA_2 mimetic) [11]. The other D304N substitution in the transmembrane domain 7 induced reduced ligand binding without disturbing surface expression. The platelet aggregation in the heterozygous patient (P1) for the D304N substitution was clearly induced by U46619 at a high concentration (10 μM), indicating that the functional defect in P1 was milder than our case [13]. This phenotype is probably as a result of residual ligand-binding capacity in the D304N variant TXA_2R. Nonetheless, these three mutations including c.167dupG dominantly inherited in terms of platelet functional defect. Recently, there is growing evidence that several GPCRs exist as homo- and hetro-oligomers and even function as oligomers, and TXA_2R dimers/oligomers have

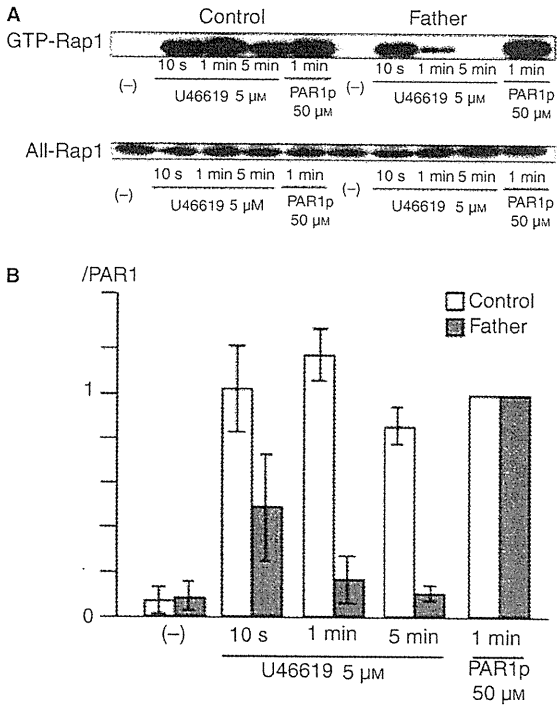


Fig. 7. Time course analysis of agonist induced Rap1 activation in platelets from the proband's father. (A) Platelet-rich plasma (PRP) (200×10^3 μL^{-1}) obtained from the proband's father or normal control was stimulated with 5 μM U46619 for 10 s, 1, and 5 min or 50 μM PAR1 for 1 min and then lysed. Rap1 activation was detected by the pull down assay using GST-RalGDS-RBD followed by incubation with rabbit anti-Rap 1B polyclonal antibody at indicated time points. Total rap1 in each lysate was detected in parallel. (B) Relative ratios as compared with Rap1 activation in response to PAR1p for 1 min are shown. Error bar represents mean ± SD of three independent experiments.

been demonstrated in platelets [19,20]. In this context, it is possible that the R60L or the D304N variant may act as a dominant negative receptor. However, the heterozygous patient described in the present study strongly suggests that the 50% reduction in the normal TXA$_2$R expression is sufficient to induce the impaired platelet response to TXA$_2$. Interestingly, Laroche *et al.* [20] demonstrated that co-expression of two TXA$_2$R signaling-deficient mutants, R60L and E2492R, resulted in rescuing of receptor signal transduction, suggesting that R60L did not show the dominant-negative effect.

We next examined the reason why 10 μM U46619-induced platelet aggregation in the proband and the father was so impaired despite the 50% expression of normal TXA$_2$R. Our initial velocity assay using 30-s incubation of FITC-PAC-1, but not the conventional binding assay with 30-min incubation of FITC-PAC-1, clearly indicated that the transient but significant $\alpha_{IIb}\beta_3$ activation (approximately 40% of control) was induced by 5 μM U46619. Similarly, the transient but significant Rap1B activation (approximately 50% of control) was also induced by 5 μM U46619. The reduction in the $\alpha_{IIb}\beta_3$ and Rap1B activation is probably as a result of the reduction in the expression of the TXA$_2$R. Nevertheless, it has been generally accepted that 50% $\alpha_{IIb}\beta_3$ activation is sufficient for normal platelet aggregation [2]. Thus, our data strongly suggested that U46619-induced sustained $\alpha_{IIb}\beta_3$ activation, but not $\alpha_{IIb}\beta_3$ activation itself, was markedly impaired in the proband and her father. It is also noteworthy that the reduction in the TXA$_2$R markedly impaired U46619-induced P-selectin (CD62p) expression in the proband. We and others have demonstrated that ADP plays a critical role in the U46619-induced $\alpha_{IIb}\beta_3$ activation via P2Y$_{12}$ [14,21]. Moreover, we have revealed that the interaction between released endogenous ADP and P2Y$_{12}$ is crucial for the sustained $\alpha_{IIb}\beta_3$ activation induced by U46619 as well as thrombin. As compared with thrombin, U46619 is less potent in inducing ADP release; approximately 5.2 μM ADP was released by 0.2 U mL^{-1} thrombin from 200×10^3 platelets μL^{-1}, whereas only 1.5 μM ADP was released by 5 μM U46619 [14]. Taken together, our data suggest that impaired ADP release may account for the impaired platelet function. To confirm this, 0.5 μM ADP was exogenously added to the father's platelets and stimulated with 5 μM U46619. The conventional binding assay as well as the initial velocity assay indicated that the small amount of ADP clearly elongated $\alpha_{IIb}\beta_3$ activation and augmented P-selectin expression (Figs 5 and 6).

The proband and her father were heterozygous for the same mutation (c.167dupG) in the *TBXA2R* gene and showed essentially the same platelet functional defects. In contrast to the proband, her father did not show any abnormal bleeding tendency. Similarly, the father heterozygous for the D304N mutation had no abnormal bleeding symptoms. Thus, heterozygosity for a mutation in the *TBXA2R* gene may not be enough for abnormal mucocutaneous bleeding, probably because of the residual response to TXA$_2$. In this context, mutations in the *TBXA2R* gene dominantly induce platelet functional defects, but not the bleeding phenotype.

Our present study presents a novel mutation responsible for the defect in the TXA$_2$R expression and reveals the mechanism responsible for the dominant inheritance of the platelet functional abnormality in response to TXA$_2$. Our findings would provide an impact on the development of novel anti-platelet agents as well as a better understanding of the TXA$_2$R abnormality.

Acknowledgements

We would like to thank Y. Otsuka (Hygo Medical College, Hyogo, Japan) for his initial examination of the proband (OSP-2). This work was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan and from the Ministry of Health, Labor and Welfare in Japan.

Disclosure of conflict of interests

The authors state that they have no conflict of interest.