Clinical Utility of Platelet Function Testing Following Non-Cardioembolic Stroke

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Objective: To verify the usefulness in selection of antiplatelet agent based on the platelet functional assays on the secondary prevention of ischemic stroke.
Methods: Platelet functional assays were performed twice for acute ischemic stroke patients at hospitalization and 1 month after. An antiplatelet agent was administered based on the results of initial assay. The alterations of platelet aggregation by antiplatelet agent were evaluated in the second assay, and the patients were subsequently divided into inhibited and invariance groups. The relationship between incidence of recurrent ischemic or hemorrhagic stroke and the alterations of platelet aggregation by each selected antiplatelet agent was assessed.
Results: Of the 585 consecutive patients, 124 were enrolled in the present study. Recurrent ischemic stroke was seen in 6 (5.3%) and 2 (18.2%) patients in the inhibited and invariance groups during the study period, respectively. In patients who were observed for more than 12 months, recurrent ischemic stroke was seen in 4 (5.0%) and 2 (33.3%) patients in the inhibited and invariance groups, respectively (p = 0.009).
Conclusions: We indicated that selection of the optimum antiplatelet agent based on the platelet functional assays for each individual patient may contribute to a reduction in the incidence of recurrence of ischemic stroke.

Key words: acute ischemic stroke, prevention, platelet functional assay, antiplatelet agent

INTRODUCTION

Stroke was the 4th leading cause of death in Japanese in fiscal year 2014, after malignant neoplasms, heart diseases, and pneumonia, according to a survey by the Ministry of Health, Labour and Welfare of Japan [1]. Since stroke patients may suffer recurrence, become bedridden, and require long-term support, effective measures to minimize recurrence are expected to have a substantial impact in reducing the costs of medical care in Japan [2].

The most common type of stroke is ischemic stroke, and a large-scale epidemiologic survey on ischemic stroke in Japan, conducted from 1999 to 2000, found that within this category, lacunar infarcts were the most common (36%), followed by atherothrombotic infarcts (31%), cardioembolic infarcts (20%), and undifferentiated types of ischemic stroke (6%) [3]. However, a large-scale stroke databank, in which more than 100,000 patients with acute stroke have been registered by the Japan Standard Stroke Registry Study Group, reported a chronological change of distribution in types of ischemic stroke from 1999 to 2012: atherothrombotic infarcts became the most common type (33.2%), overtaking lacunar infarcts (31.2%) [4].

Antiplatelet agents are commonly recommended to prevent recurrence in patients with non-cardioembolic ischemic stroke [5]. However, excessive use of antiplatelet agents may cause serious adverse events, such as digestive tract hemorrhage or intracranial hemorrhage. On the other hand, inappropriate selection of antiplatelet agents for patients may lead to lack of clinical responsiveness and recurrence of ischemic stroke. We believe that appropriate selection of antiplatelet agent(s) for each individual patient, based on monitoring of platelet function with platelet aggregation and activation assays, would be an effective strategy to minimize recurrence.

Therefore, the aim of the present study is to examine whether or not selection of antiplatelet agent(s) based on platelet functional assays, i.e., individualized treatment of patients, is effective to reduce recurrence in patients with non-cardioembolic ischemic stroke.

PATIENTS AND METHODS

Patient selection

This study was approved by the ethics committees of Tokai University (15R-099). A retrospective cohort study was conducted on consecutive patients who were hospitalized due to acute ischemic stroke in Tokai University Hospital between 2004 and 2012. Diagnosis of subtype of ischemic stroke was made by an experienced neurologist according to established criteria (NINDS-III) [6]. Only patients with non-cardioembolic ischemic stroke were enrolled in the study. All patients received platelet function assay within 7 days after hospitalization. Palatelet function was examined immediately after the blood sampling. On the basis of the

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results, an appropriate antiplatelet agent (selected from aspirin, thienopyridine derivative, and cilostazol) was newly administered or added to the previously administered antiplatelet agent as post-acute phase therapy within 10 days after hospitalization. Patients, who were in hospital for less than 1 month, or who had undergone neurovascular interventions including carotid endarterectomy, carotid artery stenting, percutaneous transluminal angioplasty, and superficial temporal artery to middle cerebral artery anastomosis, or who could not be evaluated by magnetic resonance imaging due to the presence of an indwelling metal object, were excluded from the study. Because the effectiveness of antiplatelet agents for prevention of lacunar infarcts is still controversial [7, 8], patients who were diagnosed with lacunar infarcts were also excluded from the present study. Informed consent for this study was obtained from all patients or from close relatives.

Clinical evaluation

Demographic, historical, and physical examination data of enrolled patients were collected at admission. Risk factors for atherosclerosis, including hypertension, dyslipidemia, diabetes mellitus and smoking, were evaluated by means of a medical questionnaire, physical examinations and blood tests. Hypertension was defined as blood pressure greater than or equal to 140/90 mmHg [9]. Dyslipidemia was required to meet all of the following conditions: ≥120mg/dL as low-density lipoprotein cholesterol, <40mg/dL as high-density lipoprotein cholesterol, or ≥150mg as triglyceride [10]. Diabetes mellitus was required to meet any one of the following conditions: ≥126 mg/dl as a morning fasting blood sugar level, ≥200 as the 2-hour value in the 75 g oral glucose tolerance test, ≥200 mg/dl as an after-meal blood glucose level, and ≥6.5% as hemoglobin A1c [11].

After an antiplatelet agent was administered based on the initial platelet function assay, platelet aggregation was re-examined by means of a second platelet function assay at 1 month after hospitalization, and the alteration of platelet aggregation by the administered antiplatelet agent was evaluated. Moreover, the relationship between incidence of recurrent ischemic stroke or hemorrhagic stroke and the alteration of platelet aggregation by each selected antiplatelet agent was assessed during the observation period.

Blood sampling

Blood sampling was done at around 10 a.m. in all subjects in a non-fasting condition. Blood was obtained from the antecubital vein with the aid of a light tourniquet. The first 2 ml of blood was discarded, then 4.5 ml of blood was collected slowly into a plastic syringe fitted with a 21-gauge needle (Terumo, Tokyo, Japan) and containing 0.5 ml of 3.14% sodium citrate.

Detection of platelet aggregates

Platelet aggregation was detected by means of a particle counting method using a light scattering technique [12]. Briefly, an optical device (PA-200, Kowa Co. Ltd, Japan) designed to focus on a limited area of platelet-rich plasma was used to measure the intensity of light scattered by particles passing through the area, in order to minimize multiple light scattering. The use of polystyrene spheres of different diameters confirmed that light scattering intensity increased in proportion to particle size in a suspension. Platelet activation induced by several agonists, i.e., collagen, arachidonic acid (AA), and adenosine diphosphate (ADP), resulted in higher-intensity light scattering, which correlated well with the number and size of aggregates as observed under a microscope. These findings confirmed that light scattering intensity measured with this device provides information on the number and size of aggregates in a suspension.

The concentrations of collagen, AA, and ADP were chosen, and the platelet aggregation effects were evaluated according to the algorithms as shown in Figure, which was modified from the PA-200 standard protocol.

For evaluation of platelet aggregation by collagen and ADP, 0.5 μg/ml of collagen and 1 μM of ADP were separately added, and the extent of the effect on platelet aggregation was divided into 3 classes +1 to -1 according to the proportions of aggregate sizes in the induced platelet aggregates. Class +1 was assigned if large platelet aggregates were predominantly seen in the target area, rather than small platelet aggregates. Class -1 was assigned if small platelet aggregates predominated over large platelet aggregates. In similar amounts of large and small platelet aggregates were seen in the target area, class 0 was assigned. In some cases, these classifications were visually difficult, so the platelet aggregation effect was further evaluated in all cases using a combination of 0.25 μg/ml of collagen and 0.5 μM of ADP or 1 μg/ml of collagen and 2 μM of ADP, depending on the class. In this second evaluation, the effect was again divided into 3 classes as before (subclasses +1 to -1). The cases determined to be class +1 or 0 in the initial evaluation were reevaluated with the combination of 0.25 μg/ml of collagen and 0.5 μM of ADP, and increased platelet aggregation was defined as subclass +1 that indicates increasing platelet function by collagen and ADP, while unchanged or decreased platelet aggregation was defined as subclass 0 or -1 that indicates normal platelet function by collagen and ADP. Similarly, the cases determined to be class 0 or -1 in the initial evaluation were reevaluated with the combination of 1 μg/ml of collagen and 2 μM of ADP, and increased or unchanged platelet aggregation was defined as subclass +1 or 0 that indicates normal platelet function by collagen and ADP, while decreased platelet aggregation was defined as subclass -1 that indicates suppressing platelet function by collagen and ADP.

For evaluation of platelet aggregation by AA, 500 μg/ml of AA was initially added, and the effect was divided into two groups: large or small platelet aggregate formation. To confirm the results, cases in the large platelet aggregate group were reevaluated using 250 μM of AA. In this second evaluation, the effect was again divided into subgroups of large and small platelet aggregate formation. Finally, the large and small platelet aggregate formation subgroups were defined as increasing and normal platelet functions by AA, respectively. Cases in the small platelet aggregate group in the initial evaluation were considered to show

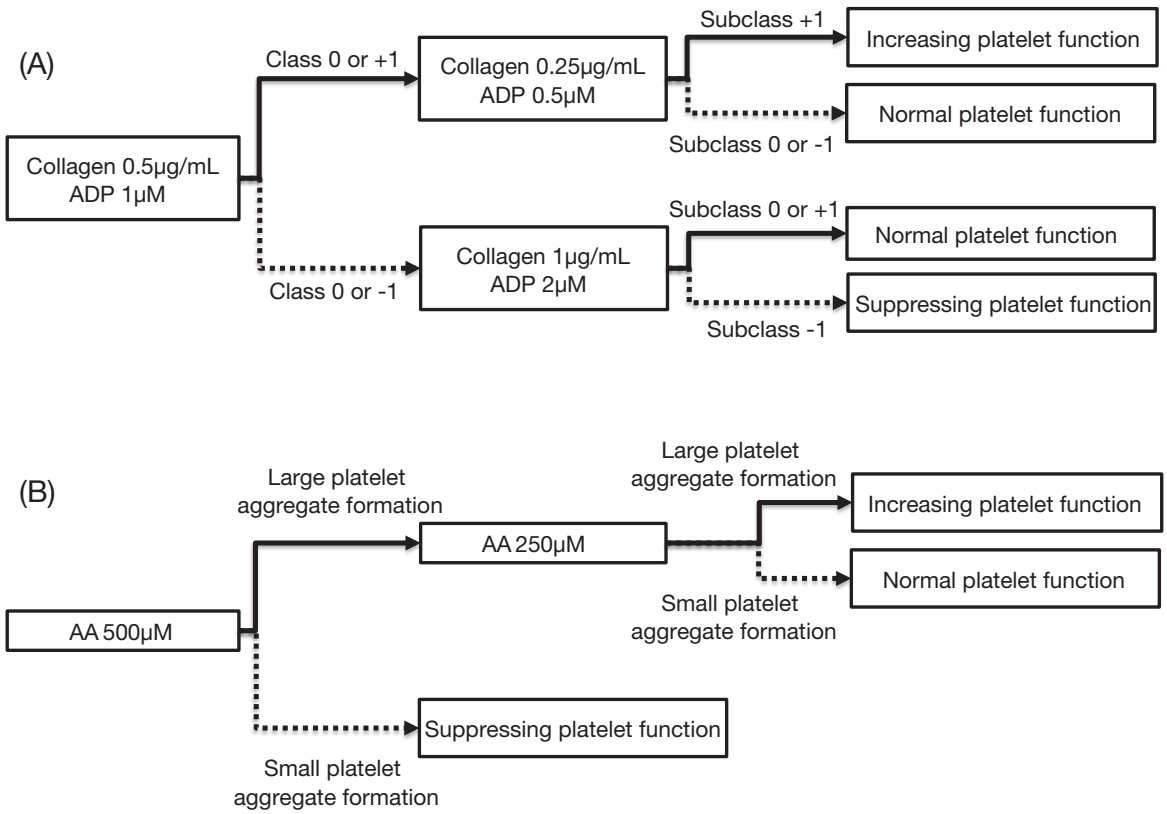

Figure Algorithms for detection of platelet aggregation by collagen and adenosine diphosphate (ADP) (A), and arachidonic acid (AA) (B).

(A) For evaluation of platelet aggregation by collagen or ADP, 0.5 μg/ml of collagen or 1 μM of ADP were initially added. Class 0 or +1 in proportions of aggregate sizes in the initial evaluation was reevaluated with the combination of 0.25 μg/ml of collagen and 0.5 μM of ADP, and increased platelet aggregation was defined as subclass +1 that indicates increasing platelet function by collagen and ADP, while unchanged or decreased platelet aggregation was defined as subclass 0 or -1 that indicates normal platelet function by collagen and ADP. Similarly, class 0 or -1 in proportions of aggregate sizes in the initial evaluation was reevaluated with the combination of 1 μg/ml of collagen and 2 μM of ADP, and increased or unchanged platelet aggregation was defined as subclass +1 or 0 that indicates normal platelet function by collagen and ADP, while decreased platelet aggregation was defined as subclass -1 that indicates suppressing platelet function by collagen and ADP.

(B) For evaluation of platelet aggregation by AA, 500 μg/ml of AA was initially added. The large aggregate formation group in platelet size in the initial evaluation was reevaluated using 250 μM of AA. The large and small platelet aggregate formation subgroups in the second evaluation were defined as increasing and normal platelet functions by AA, respectively. Cases in the small platelet aggregate group in the initial evaluation were considered to show suppressing platelet function by AA.

suppressing platelet function by AA.

Selection of antiplatelet agent and evaluation of the effect on platelet aggregation

Different antiplatelet agents generally inhibit different factor(s) associated with platelet aggregation [13, 14]. Therefore, patients who showed increased platelet aggregation in response to collagen and/or AA were given aspirin as a post-acute phase therapy. On the other hand, patients who showed increased platelet aggregation in response to ADP and AA were administered thienopyridine derivative and cilostazol as post-acute phase therapies, respectively.

To evaluate the effect of the selected antiplatelet agent on platelet aggregation, all patients were re-examined using the same platelet functional assay at 1 month after hospitalization. In patients who were given aspirin, it was considered that the platelet aggregation was inhibited if suppression of platelet aggregation by both collagen and AA was seen. Similarly, platelet aggregation was considered to be inhibited by thienopyridine derivative and cilostazol if suppression of platelet aggregation by ADP and AA, respectively, was observed. On the other hand, it was defined that platelet aggregation was invariable if no suppression by the antiplatelet agent was observed. Based on the obtained changes of platelet aggregation by antiplatelet agents at 1 month after hospitalization, all patients were divided into the inhibited or invariance group.

Statistical analysis

The chi-square test was used for categorical data, and the Mann-Whitney and Kruskal-Wallis tests were used for interval data. Statistical analyses were performed using SPSS 23.0 (SPSS, Inc. Chicago, IL, USA). Data were presented as mean ± SD. The significance level was set at $P < 0.05$.

Table 1 Clinical features of patients in the present study

	Total (N = 124)	Classification of acute ischemic stroke		
		Atherothrombosis (N = 56)	TIA (N = 9)	Others (N = 59)
Age, years (mean ± SD)	67.5±11.3	68.5±10.4	61.8±13.0	67.5±11.8
Female, n (%)	41 (66.9)	16 (28.6)	4 (44.4)	21 (35.6)
Co-existing risk factors for atherosclerosis, n (%)				
Hypertension	92 (74.2)	38 (67.9)	6 (66.7)	48 (81.4)
Dyslipidemia	72 (58.1)	35 (62.5)	5 (55.6)	32 (54.2)
Diabetes mellitus	37 (29.8)	16 (28.6)	2 (22.2)	19 (32.2)
Smoking	64 (51.6)	28 (50.0)	5 (55.6)	31 (52.5)
Types of antiplatelet agent, n (%)				
Aspirin	45 (36.3)	21 (37.5)	3 (33.3)	21 (35.6)
Clopidogrel	48 (38.7)	19 (33.9)	3 (33.3)	26 (44.1)
Cilostazol	8 (6.5)	3 (5.4)	1 (11.1)	4 (6.8)
Aspirin + Clopidogrel	3 (2.4)	2 (3.6)	1 (11.1)	0 (0.0)
Aspirin + Cilostazol	19 (15.3)	11 (19.6)	1 (11.1)	7 (11.9)
Clopidogrel + Cilostazol	1 (0.8)	0 (0.0)	0 (0.0)	1 (1.7)

RESULTS

Clinical characteristics

During the study period, platelet aggregation tests were performed for 585 consecutive patients with acute ischemic stroke. Of these, 124 fulfilled the inclusion criteria and the platelet aggregation test was repeated 1 month after the initial test. Clinical characteristics of the 124 patients are summarized in Table 1. The age at hospitalization was 67.5 ± 11.3 years (mean ± SD). Among the co-existing risk factors for atherosclerosis, hypertension was seen in a high proportion of patients. Based on the results of the initial platelet aggregation test, we selected the optimal antiplatelet agent as post-acute phase therapy for prevention of recurrent ischemic stroke. If indicated, a different type of antiplatelet agent was newly added to the previously administered antiplatelet agent after the initial platelet aggregation test. This study included 56 patients with atherothrombosis, 9 with TIA, and 59 with other types of acute ischemic stroke. No significant differences were observed in age, gender distribution, proportions of co-existing risk factors for atherosclerosis, and proportions of types of post-acute phase therapy among these 3 groups.

Relationship of clinical characteristics to changes in platelet aggregation

After one month of post-acute phase therapy with the antiplatelet agent selected based on the result of the initial platelet aggregation test, we re-examined platelet aggregation. The numbers of patients who showed inhibition and who showed unchanged platelet aggregation were 113 and 11, respectively (Table 2). No significant differences in age, gender distribution, proportion of co-existing risk factors for atherosclerosis, and proportions of types of post-acute phase therapy were observed. Follow-up durations after hospitalization were 34.4 and 19.6 months in the inhibited and invariance groups, respectively. There was no significant difference in follow-up duration between the two groups.

Recurrence of ischemic and hemorrhagic strokes in the inhibited and invariance groups

The numbers of patients with recurrent ischemic and hemorrhagic strokes in the inhibited and invariance groups are shown in Table 3. Recurrent ischemic stroke was seen in 6 (5.3%) and 2 (18.2%) patients in the inhibited and invariance groups during the study period, respectively. This difference was not statistically significant. We next subdivided the follow-up duration into 2 categories: less than or equal to 12 and more than 12 months. In patients who were followed up for 12 months or less, two (6.1%) developed recurrent ischemic stroke in the inhibited group, and none did so in the invariance group. This difference is also not statistically significant. However, in patients who were observed for more than 12 months, recurrent ischemic stroke was seen in 4 (5.0%) and 2 (33.3%) patients in the inhibited and invariance groups, respectively, and this difference was significant ($p = 0.009$).

During the study period, none of the patients showed hemorrhagic stroke in the inhibited group, and 1 (9.1%) patient developed hemorrhagic stroke in the invariance group (not significant). However, the one hemorrhagic stroke in the invariance group occurred during follow-up of 12 months or less, while none was seen in patients in the inhibited group who were observed during those follow-up period, and this difference is significant ($p = 0.030$). Hemorrhagic stroke did not occur in any patient followed-up for more than 12 months in either group.

DISCUSSION

The present results indicated that inhibition of platelet aggregation using a specific antiplatelet agent selected on the basis of evaluation by platelet func-

Table 2 Clinical characteristics in inhibited and invariance groups

	Changes in platelet aggregation by anti-platelet	
	Inhibited	Invariance
No. of patients	113	11
Age, years (mean ± SD)	67.6 ± 11.2	67.0 ± 13.2
Female, n (%)	37 (32.7)	4 (36.4)
Classification of primary ischemic stroke, n (%)		
Atherothrombosis	51 (45.1)	5 (45.5)
TIA	8 (7.1)	1 (9.0)
Others	54 (47.8)	5 (45.5)
Post-acute phase therapy, n (%)		
Aspirin	40 (35.4)	5 (45.5)
Clopidogrel	43 (38.1)	5 (45.5)
Cilostazol	8 (7.1)	0 (0)
Aspirin + Clopidogrel	3 (2.7)	0 (0)
Aspirin + Cilostazol	18 (15.9)	1 (9.1)
Clopidogrel + Cilostazol	1 (0.9)	0 (0)
Follow-up duration, months (mean ± SD)	34.4 ± 27.0	19.6 ± 21.6

Table 3 Numbers of patients with recurrent ischemic or hemorrhagic stroke in inhibited and invariance groups

Changes in platelet aggregation by anti-platelets			Recurrent ischemic stroke n, (%)	Hemorrhagic stroke n, (%)
Inhibited group	Follow-up duration	Overall (n = 113)	6 (5.3)	0 (0.0)
		≤ 12 months (n = 33)	2 (6.1)	0 (0.0)
		> 12 months (n = 80)	4 (5.0)	0 (0.0)
Invariance group	Follow-up duration	Overall (n = 11)	2 (18.2)	1 (9.1)
		≤ 12 months (n = 5)	0 (0.0)	1 (20.0)¶
		> 12 months (n = 6)	2 (33.3)†	0 (0.0)

†p = 0.009, $\chi 2$ test. ¶p = 0.030, $\chi 2$ test.

tional assay, together with careful evaluation of the factor(s) contributing to platelet aggregation in each individual, may be an effective strategy for long-term prevention of recurrent ischemic stroke. There have been several reports on the recurrence rate of ischemic stroke [15–17]. However, our work is the first to indicate that evidence-based selection of the optimum antiplatelet agent for each individual patient may contribute to a reduction in the incidence of recurrence of ischemic stroke.

On the basis of the initial platelet function assays, an appropriate antiplatelet agent as post-acute phase therapy has been selected. However, platelet aggregation was invariance in 11 patients of the present study, and the lasting activation of platelet function may lead to the recurrence of ischemic stroke. It has been distinctly established that aspirin and thienopyridine derivative are effective for secondary prevention of ischemic stroke [18–21]. On the other hand, there also exist patients who have a platelet function resistance to aspirin and thienopyridine derivative [22, 23]. We demonstrated in the present study that inhibitions of platelet aggregation by using an appropriate antiplatelet agent based on the platelet functional assays provide reliable secondary prevention of ischemic stroke in a long-term period. Additionally, it is also possible to detect the patient who shows invariance of platelet aggregation by using antiplatelet agent suggesting the resistance to antiplatelet therapy by platelet functional assays. Preventing the occurrence of ischemic stroke in patient resistance to antiplatelet therapy is a crucial issue, and reevaluation of platelet functional assays may be useful to indicate further strict treatment of co-existing risk factors for atherosclerosis in those patients.

In the present study, no significant difference in the recurrent rate of ischemic stroke between the inhibited and invariance groups during 12-month follow-up period was present, whereas the significant preven-

tion for recurrent ischemic stroke was found only in the inhibited group during a long-term period. The emergence of recurrent ischemic stroke is generally expected to show an increasing tendency according to lengthening of follow-up period. However, we considered that the platelet functional assays leading to optimizing antiplatelet therapy achieved the prevention of recurrent ischemic stroke in a long-term period of the present study. Several large scale studies have reported the annual recurrent rate of ischemic stroke under the circumstance of antiplatelet therapy was 2.8–3.8% [16, 17]. The recurrence rate of ischemic stroke in the inhibited group of the present study was 5.3% in mean follow-up period of 34 months. Unlike in previous studies, we excluded the patients with lacunar infarcts from the present study sample. These differences in each study method may results in a variety of recurrence rate of ischemic stroke.

The annual rate of hemorrhagic stroke under the circumstance of antiplatelet therapy has been reported variously in countries and the types of platelet agent. Large-scale randomized studies that aimed to prevent recurrent ischemic stroke have reported the annual rate of hemorrhagic stroke as 0.15–0.43% in Europe and 1.00–1.89% in Asia with aspirin therapy, as 0.18–0.47% in Europe and 0.20–0.45% in Asia with thienopyridine derivative therapy, and as 0.27–0.42% in Asia with cilostazol therapy [24]. In the present study, one patient (0.81%) developed intracranial hemorrhage in the all enrolled patients, and besides, none of the patients showed hemorrhagic stroke in the inhibited group. Therefore, we considered that appropriate selection of antiplatelet agents using platelet functional assays provided the preventions not only recurrent ischemic stroke but also undesirable hemorrhagic stroke.

Some factors which may affect our results of platelet function should be taken into consideration. First, antithrombotic agents such as thromboxane A2 synthase inhibitor, ozagrel, are sometimes given before the measurement of platelet function in acute phase of non-cardioembolic stroke. Drug interview form of ozagrel indicates that T 1/2 of intravenous administration of ozagrel is estimated to 0.06 ± 0.04 hours (http://www.kissei.co.jp/di/vcdb/da/di/xa/). Therefore, ozagrel, which is usually given at 6:00 A.M. in Tokai University hospital, is less influenced on blood sampling for platelet function assay at 10 a.m. in all subjects in this study. Next, serial changes of platelet activation after ischemic stroke remain unclear. Previous reports indicated that platelet function is activated at 7 or 10 days after onset of ischemic stroke and TIA, but serial changes of platelet function in a week after onset have not been precisely examined [25–27]. Even if activation of platelet function varies within a week after onset, we measured platelet function at same timing after onset between two groups, indicating no serious effect is speculated.

The principal limitation of this study was the small sample size. The effect of other factors such as risks of atherosclerosis affecting recurrence of ischemic stroke excepting antiplatelet activation has not also been evaluated. Additionally, the methodology of platelet functional assays in the present study is not of which has been generally established, whereas a battery of platelet functional assays and the subsequently obtained results regarding secondary prevention of stroke indicated the reliability in the methods of the present platelet functional assays. Further large-scale and multicenter studies are required for additional validation of the platelet functional assays and the utility for secondary prevention of stroke in patients with antiplatelet therapy.

In conclusion, this is the first cohort study to verify the appropriate selection of antiplatelet agents by means of platelet functional assays. We concluded that inhibition of platelet aggregation using a specific antiplatelet agent selected on the basis of evaluation by platelet functional assays, together with careful evaluation of the factors contributing to platelet aggregation in each individual, may be an effective strategy for long-term prevention of recurrent ischemic stroke.

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