Assessment of an ELISA Kit for Platelet-Derived Microparticles by Joint Research at Many Institutes in Japan

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Aim: Platelet-derived microparticles (PDMPs) play roles in normal hemostatic responses to vascular injury because they possess prothrombinase activity. Although the most widely used method for studying PDMP is flow cytometry, we previously developed an enzyme-linked immunosorbent assay (ELISA) method as an easier and more reproducible PDMP assay. The purpose of this study was to use various clinical settings to verify whether this ELISA method can produce equivalent results to flow cytometry for PDMP.

Methods: We performed a large-scale clinical study for various thrombotic and subatherothrombotic diseases using an ELISA kit. The study group included 692 patients with cerebral infarction, heart failure, acute coronary syndrome or diabetes mellitus.

Results: When baseline PDMP values in the various diseases were compared with those in healthy controls, significant differences were noted in all cases. There were significantly elevated levels of PDMPs in diabetic patients with complications but no thrombosis. When baseline PDMP values in cerebral infarction were compared within the subclassifications, atheroma and other types of infarction exhibited significantly elevated PDMP levels compared with lacunar infarction. Cerebral infarction exhibited a significant change in PDMPs after therapy compared with the baseline (before therapy), but not in acute coronary syndrome and heart failure. The ELISA method exhibited results almost identical to flow cytometry for PDMP in various atherothrombotic diseases.

Conclusion: Although further examinations to evaluate the therapeutic usefulness of these diseases are necessary, ELISA kits possibly represent a new tool for PDMP related to atherothrombosis.


Key words; PDMP, ELISA kit, Atherothrombotic diseases, Diabetic complication, Cerebral infarction

Introduction

Microparticles (MPs) are small membrane vesicles that are released from many different cell types by a process of exocytic budding of the plasma membrane¹. Because MPs disseminate various bioactive effectors originating from the parent cells, they can alter vascular function and may induce biological responses involved in the vascular system². Although MPs are released from various cells¹,³,⁴, most MPs in human blood originate from platelets. Platelet-derived microparticles (PDMPs) play roles in normal hemo-
static responses to vascular injury because they possess prothrombinase activity\(^1\). PDMPs are also released from platelets following physical stimulation under various conditions\(^2\)–\(^5\)^, and it is considered that PDMPs contribute to the development of thrombotic complications in pathologic states associated with increases in their blood concentrations\(^9\)–\(^10\).

The most widely used method for studying PDMP is flow cytometry. This method has a lot of advantages, such as simplicity and a wealth of information that can be gleaned from the population of samples; however, this method is too expensive. In addition, standardization of PDMP quantity has not yet been perfected, although this method has begun\(^11\). We previously developed an enzyme-linked immunosorbent assay (ELISA) method as an easier and reproducible PDMP assay\(^12\)–\(^13\). This method will hopefully contribute toward understanding the participation of PDMPs in clinical settings\(^14\); however, the usefulness of this kit has not yet been perfected. The purpose of this study was to use various clinical settings to verify whether an ELISA method can achieve results equal to the results from flow cytometry obtained for PDMP. To the best of our knowledge, this is the first study to evaluate the clinical significance of PDMPs using an ELISA method in multiple institutions.

**Methods**

**Measurements of PDMPs**

The PDMP ELISA kit\(^12\)–\(^15\) was obtained from JIMRO Co. Ltd. (Gunma, Japan). The kit used two monoclonal antibodies against glycoproteins CD42b and CD42a. One U/mL of PDMPs in this ELISA kit was defined as the amount of PDMPs obtained from 24,000 solubilized platelets/mL. Blood samples were collected from peripheral veins into vacutainers containing EDTA-ACD (NIPRO Co. Ltd., Osaka, Japan) using 21-gauge needles to minimize platelet activation. The samples were gently mixed by turning the tubes upside down once or twice and then kept at room temperature for the maximum period of 2–3 hours. Immediately after centrifugation at 8,000 \(g\) for 5 minutes, 200 \(\mu\)L was collected from the upper-layer supernatant of the 2 mL samples to avoid contamination by platelets. The collected samples were stored at \(-40^\circ\)C until analysis. PDMP levels were measured twice and mean values were recorded. Furthermore, some basic studies were carried out prior to this measurement using clinical specimens.

**Study Population**

The control group was extracted from the data of normal subjects as previously reported\(^14\). Patients were prospectively selected including 692 patients with cerebral infarction, heart failure, acute coronary syndrome or diabetes mellitus (Table 1). Between April 2006 and October 2007, patients were selected from patients admitted to each institute (see first page and other participants). None had had inflammatory diseases within the previous three months, were pregnant or had clinically detectable renal, hepatic, infectious, thyroid or malignant diseases. The study protocol was approved by the Institutional Review Boards (IRBs) of all the institutes and written informed consent was obtained from each patient prior to the start of the study.

<table>
<thead>
<tr>
<th>Table 1. Baseline characteristics of the study population</th>
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<tbody>
<tr>
<td><strong>Patients characteristics</strong></td>
</tr>
<tr>
<td>Total procedures</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Gender (male/female)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Cerebral infarction</td>
</tr>
<tr>
<td>Gender (male/female)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Heart failure</td>
</tr>
<tr>
<td>Gender (male/female)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Acute coronary syndrome</td>
</tr>
<tr>
<td>Gender (male/female)</td>
</tr>
<tr>
<td>Age, y</td>
</tr>
</tbody>
</table>

**Study Design**

We examined 3 groups of patients as the subjects of the study, as described below.

The first group comprised a nonthrombotic diabetes group. The subjects in this group had type 1 or type 2 diabetes mellitus with no thrombotic events until the current time. Patients having a serum creatinine level below 3.0 mg/dL were enrolled. The complications analyzed were nephropathy, retinopathy, neuropathy, hypertension and hyperlipidemia. Proteinuria was evaluated by quantitative analysis.

The second group comprised a cerebrovascular disorder group. The subjects in this group had had a new cerebral infarction within 48 hours of symptom appearance and exhibited a recognized infarction sign on imaging. Furthermore, only patients exhibiting symptoms for longer than 1 hour were enrolled. The disease type classifications were divided into four groups of lacunar, atheroma, cardiogenic and others. The third group comprised a cardiovascular disorder group. The subjects in this group had heart fail-
ure or acute coronary syndrome. Patients with acute coronary syndrome were restricted to cases within 48 hours of symptom appearance. Acute coronary syndrome was classified into two subgroups of ST-elevation and non-ST-elevation. Heart failure was classified as acute type or a crisis of the chronic type. Heart failure was further classified into two subgroups of ischemic and non-ischemic type.

**Effect of the Therapy**

We collected samples before and after therapy from the cerebral infarction, acute coronary syndrome and heart failure groups to examine whether the ELISA kit can recognize the role of PDMP in the therapeutic effects of ASA, ticlopidine, clopidogrel or cilostazol. The sampling time of blood collection was at admission or pretreatment and at 1 month after treatment. PCI was performed according to standard techniques. All patients received a stent (bare metal or drug eluting), followed by aspirin and ticlopidine or clopidogrel administration. An initial blood sample was obtained before intervention, and then subsequent samples were obtained after 30 days. Heart failure was treated by diuretic or cardiac drugs.

**Statistical Analysis**

All parameters examined in the present study were expressed as the mean ± SD. In all tests, values of \( p < 0.05 \) were considered to indicate statistical significance. Data were analyzed by an n-ary decision tree with chi-square automatic interaction detection (CHAID) as an additional algorithm. Between-group comparisons were carried out with the Kruskal-Wallis test and a post-hoc test using Dunnett’s multiple comparisons. Within-group differences were determined by the Wilcoxon rank sum test for paired values. Correlation testing was performed using the Spearman and Kendall rank correlation coefficients.

**Results**

**Accuracy of ELISA for PDMP Measurement**

It is clear that the nature of the anticoagulant added during blood collection can markedly affect platelet function; therefore, if the appropriate anticoagulant is not chosen, platelets can be activated easily. We observed that platelet activation by centrifugation resulted in a clear elevation of PDMPs in whole blood collected into citrate compared with blood anticoagulated with EDTA-ACD (Fig. 1). Next, we evaluated the appropriate centrifugal conditions to measure PDMP by changing the gravitational acceleration and centrifugal time. The stabilizing values of PDMPs were obtained at a centrifugal acceleration of 6,000 g and centrifugal time of 3 min (Fig. 2); therefore, we determined the standard method of centrifugal acceleration and time, 8,000 g and 5 min, respectively. Furthermore, we investigated the optimal storage conditions after the separation of PDMP samples at −4°C and −80°C. It was possible to preserve samples at equal to or less than −40°C (Fig. 3). As illustrated in Fig. 4, the PDMP ELISA kit is a sandwich ELISA system using anti-GPIX and anti-GPIb monoclonal antibodies. The performance of this kit has obtained suitable reproducibility such as simultaneous CV (1.1–4.0%) and daily CV (5.2–8.8%).

**Examination Using Clinical Samples of Various Diseases**

Patients with various diseases and those on certain medications that affect the generation of PDMPs were excluded from the disease groups; for example, pregnancy, cancer disease, thyroid disease, use of steroids, hormone replacement therapy and anti-thrombotic treatment were exclusion criteria. Since 35 patients were excluded based on this criteria, 594 of 629 patients were analyzed. When the baseline PDMP levels in the various diseases were compared with those in healthy controls, significant differences were noted in diabetes mellitus, cerebral infarction, acute coronary syndrome and heart failure (Fig. 5). These differences were also significant using the Kruskal-Wallis test (\( p < 0.001 \)) and post-hoc test by Dunnett’s multiple comparisons (\( p < 0.05 \)).
Analysis of Diabetes Mellitus

Table 2 shows the relationships between PDMPs and diabetes mellitus. There were no significant differences in PDMP levels between males and females. On the other hand, complications such as nephropathy, retinopathy or neuropathy exhibited significant elevated levels of PDMPs, but hypertension or hyperlipidemia was not associated with PDMP elevation. PDMPs in patients with positive proteinuria exhibited a significant elevation in levels compared to those in patients with negative proteinuria (Table 3); however, PDMPs did not show any significant correlation with blood urea nitrogen or creatinine (data not shown).

Analysis of Cerebral Infarction, Acute Coronary Syndrome and Heart Failure

Table 4 shows the baseline PDMP levels in cerebral infarction. There were no significant differences
in PDMP levels between males and females. When baseline PDMP levels for cerebral infarction were compared within the classifications, atheroma and other types of infarction exhibited significantly elevated PDMP levels compared with lacunar infarction; however, cardiogenic infarction did not show such significant differences.

Table 5 shows the baseline PDMP levels for heart failure and acute coronary syndrome. There were no significant differences in PDMP levels between males and females for heart failure, but a significant difference was apparent for acute coronary syndrome. Furthermore, the classifications for heart failure (ischemic vs. nonischemic) and acute coronary syndrome (ST-elevation vs. non-ST-elevation) exhibited no significant differences.

### Effects on the Treatment

Cerebral infarction exhibited a significant change in PDMPs after therapy compared with the baseline (before therapy) (Fig. 6); however, heart failure and acute coronary syndrome exhibited no significant changes in PDMPs after therapy compared with the baseline (Fig. 6).

### Discussion

We first examined the reproducibility of the ELISA kit to verify whether this kit can be useful for research on PDMPs in various clinical settings. We conquered several problems such as anticoagulation, centrifugation and preservation optimization, and finally achieved suitable reproducibility with this ELISA kit.

Next, our mission was to clinically apply the ELISA kit. The most important point in the present study was to test this kit in many institutions. Our first application in the clinical setting was for diabetes mellitus, in which PDMPs may contribute to a prothrombotic state and promote the progression of atherosclerosis, finally resulting in atherosclerosis that can
be related to abnormalities in the coagulation and fibrinolytic pathways, as well as in platelet function. In fact, some studies regarding the potential roles of PDMPs in diabetic complications have been carried out using flow cytometric analysis.\[^{16-22}\] The present study using an ELISA method produced similar results. In particular, the most interesting piece of evidence obtained in the present study is the correlation between PDMPs and diabetic complications. There were significantly elevated levels of PDMPs in diabetes with nephropathy, retinopathy or neuropathy. PDMPs in patients with positive proteinuria also exhibited a significant elevation in levels compared to those in patients with negative proteinuria. This result suggests the possibility that PDMPs are associated with potential for renal dysfunction, although PDMPs were not correlated with blood urea nitrogen or creatinine. PDMPs were previously reported to be correlated with soluble E-selectin, which plays a role in the acceleration of diabetic macroangiopathy.\[^{23, 24}\] Furthermore, Omoto \textit{et al.}\[^{18}\] placed special emphasis on the significance of P-selectin-positive platelets and PDMPs in diabetic nephropathy. Thus, the present data support these previous reports using flow cytometry.

Cerebral infarction and acute coronary syndrome were chosen to examine the usefulness of the ELISA kit for PDMP measurement in diseases which cause thrombosis. Lee \textit{et al.}\[^{25}\] previously reported that transient ischemic attacks or lacunar infarctions had sig-

**Table 2. PDMPs and diabetes mellitus**

<table>
<thead>
<tr>
<th>Gender</th>
<th>n</th>
<th>PDMPs (IU/mL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>135</td>
<td>12.3 ± 11.2</td>
<td>0.0912</td>
</tr>
<tr>
<td>Female</td>
<td>123</td>
<td>13.5 ± 10.9</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. PDMPs and proteinuria in patients with diabetes**

<table>
<thead>
<tr>
<th>Proteinuria</th>
<th>PDMPs (IU/mL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>9.3 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>11.8 ± 7.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>+</td>
<td>14.9 ± 7.2</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>++</td>
<td>17.8 ± 8.7</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>+++</td>
<td>13.5 ± 6.3</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

**Table 4. Baseline PDMPs of the subgroup population in cerebral infarction**

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number (n)</th>
<th>PDMP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>146 (60.6)</td>
<td>15.9 ± 18.2</td>
<td>0.3966</td>
</tr>
<tr>
<td>Female</td>
<td>98 (40.4)</td>
<td>13.4 ± 12.8</td>
<td></td>
</tr>
</tbody>
</table>

**Classification**

<table>
<thead>
<tr>
<th>Infarction</th>
<th>number (n)</th>
<th>PDMP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lacunar</td>
<td>127 (52.5)</td>
<td>12.1 ± 15.3</td>
<td></td>
</tr>
<tr>
<td>Atherome</td>
<td>74 (30.6)</td>
<td>16.3 ± 17.9</td>
<td>0.0186*</td>
</tr>
<tr>
<td>Cardiogenic</td>
<td>28 (11.6)</td>
<td>12.4 ± 4.8</td>
<td>0.1980</td>
</tr>
<tr>
<td>Other</td>
<td>13 (5.4)</td>
<td>26.9 ± 28.4</td>
<td>0.0007*</td>
</tr>
</tbody>
</table>

\(p\) value: Gender (vs. Male) Classification (vs. Lacunar infarction)

*: Abnormality of blood coagulation system or blood vessel.

*: statistically significant
nificantly elevated PDMP levels, and that PDMP levels were higher in patients with small-vessel stroke than in those with large-vessel stroke. This report using flow cytometry suggests the significance of PDMP in cerebral infarction. In the present study using this ELISA kit, the significance of PDMPs in cerebral infarction was also very informative, especially the findings concerning disease classifications. For example, atheromatous infarction exhibited a significant elevation of PDMPs compared with lacunar infarction. Recently, Tan \textit{et al.}\cite{26} suggested that PDMPs play roles in all stages of the pathogenesis of ischemic stroke. The present results appear to confirm these previous reports using flow cytometry. On the other hand, the effects of acute coronary syndrome were subtle. Involvement of PDMPs in acute coronary syndrome has been reported in previous studies\cite{5,13,27-30}. Katopodis \textit{et al.}\cite{30} evaluated platelet calcium homeostasis and activation markers in patients with coronary artery disease, resulting in significantly higher PDMP levels in patients with acute coronary syndrome than in controls. Thus, the significance of PDMPs for atherothrombotic events is also very important in acute coronary syndrome. The present results using the

Table 5. Baseline PDMPs of the subgroup population in heart failure and acute coronary syndrome

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>PDMP</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart failure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>33 (67.3)</td>
<td>15.8 ± 13.3</td>
<td>0.5730</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>16 (32.7)</td>
<td>12.9 ± 12.7</td>
<td>0.8992</td>
</tr>
<tr>
<td>Ischemic, n (%)</td>
<td>19 (38.8)</td>
<td>14.1 ± 13.9</td>
<td></td>
</tr>
<tr>
<td>Unischemic, n (%)</td>
<td>30 (61.2)</td>
<td>14.7 ± 13.1</td>
<td></td>
</tr>
<tr>
<td><strong>Acute coronary syndrome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>26 (86.7)</td>
<td>33.2 ± 51.5</td>
<td>0.0081*</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>4 (13.3)</td>
<td>115.2 ± 68.3</td>
<td></td>
</tr>
<tr>
<td>ST-elevation, n (%)</td>
<td>24 (80.0)</td>
<td>51.7 ± 67.4</td>
<td></td>
</tr>
<tr>
<td>Non ST-elevation, n (%)</td>
<td>6 (20.0)</td>
<td>19.9 ± 17.5</td>
<td></td>
</tr>
</tbody>
</table>

\(p\) value: Gender (vs. Male) Classifications (vs. Ischemic in heart failure or vs. ST-elevation in acute coronary syndrome)

*: statistically significant

Fig. 6. Changes in PDMP levels in patients after therapy relative to the baseline. Bars are the shown as means ± SD, with \(p\) values relative to the corresponding baseline values (before vs. after).
ELISA kit support these previous reports. Our final interesting finding from the present study was whether the ELISA kit reflects the therapeutic effects of PDMPs. In cerebral infarction, PDMP levels were significantly decreased after therapy compared with the levels before therapy. There have been a few reports of a significant improvement in PDMPs in cerebral infarction after anti-thrombotic therapy\(^{31}\). Our results appear to be important, since understanding the mechanisms of PDMP release may aid the development of useful therapies and the prevention of stroke; however, PDMP levels might decrease independently from the introduced therapy, because our follow up point was limited to one month after therapy. In the future, a more suitable follow-up point will be needed to bolster our conclusion.

We obtained useful results for the cerebral infarction group, but were nevertheless unable to detect improvements in PDMP levels after therapy for both heart failure and acute coronary syndrome. Gawaz et al.\(^{27}\) examined various aspects of platelet function in patients with acute myocardial infarction undergoing PCI. They concluded that platelet activation is significantly enhanced soon after direct PCI as reflected by increased platelet consumption and PDMP formation. In addition, Merten et al.\(^{32}\) reported that PDMPs bind to the subendothelial matrix in vitro and in vivo and can act as a substrate for further platelet binding. Thus, these previous reports using flow cytometry suggest further elevated PDMP levels after PCI. In the present study using the ELISA method, we could not observe a significant improvement of PDMP levels after PCI. On the other hand, Namba et al.\(^{29}\) reported that PDMP levels after PCI are full of variety and higher PDMP levels at discharge were associated with poorer clinical outcomes at 1 year using the ELISA method. Although Nomura et al.\(^{13}\) reported that PDMP levels in acute coronary syndrome were significantly decreased after PCI, there is a possibility that these might have been rare cases because thrombus might not have formed afterwards. At present, it is difficult to interpret the precise cause of the changes in PDMP levels before and after therapy in acute coronary syndrome. One reason may be the presence of subpopulations of PDMPs. Van der Zee et al.\(^{28}\) reported that PDMP subpopulations reflect the platelet activation status more effectively than the total number of PDMPs. Furthermore, PDMP subpopulations differ significantly between the contents of plaque and peripheral blood\(^{33}\); however, in the present study, we could not investigate these subpopulations of PDMPs. Another reason might be differences in the devices used, such as a bare metal stent and drug-eluting stent. The kind of stent may affect platelet activation after PCI. Further investigations are therefore necessary to reach a conclusion regarding the significance of PDMPs after PCI.

It is now well established that activated platelets play roles in the evolution of atherosclerosis, from the initiation of the fatty streak through the progression of atheromatous plaque to the final atherothrombotic events\(^{34-38}\). More recently, increasing evidence has suggested that the roles of platelets in atherosclerosis may at least be partially mediated by the production of PDMPs\(^{6, 11, 39-42}\). For example, at high shear stress, PDMP rolling enables the delivery of RANTES to the inflamed endothelium, thus favoring monocyte adhesion and plaque infiltration. Other types of MPs, leukocyte- or endothelial cell-derived, have also been investigated in patients with atherothrombosis\(^{1, 3, 4, 43}\). It is possibly thought that these MPs are somewhat related\(^{44}\); however, PDMPs must be the most important participant in atherothrombosis because they are quantitively abundant and are generated by activated platelets. Indeed, research into PDMPs frequently uncovers new evidence\(^{1, 45, 46}\). We believe that this ELISA method can contribute to ongoing research into PDMP.

In conclusion, this ELISA method produced similar results to flow cytometry for PDMP in various atherothrombotic diseases. Although further examination concerning the usefulness for therapeutic evaluation of these diseases is necessary, ELISA kits may represent a new tool for PDMP changes related to atherothrombosis.

**Acknowledgment**

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**Disclosure of Conflicts of Interest**

The authors state that they have no conflicts of interest.
Other Participants

In addition to the authors, the following institutions and investigators participated in this study: N. Inami (Suzuran Hospital, Kobe); E. Murakami (Toho University Omori Medical Center, Tokyo); Y. Hasegawa, K. Yamada, R. Oikawa (St.Marianna University School Of Medicine, Kanagawa); H. Satoh (Hamamatsu University School of Medicine, Shizuoka); T. Inoue (Saga Medical School Faculty of Medicine, Saga); A. Kashiwagi, A. Kishi, S. Araki (Shiga University of Medical Science, Shiga); Y. Ikeda, H. Iwaza (Tokyo Medical University Hachioji Medical Center, Tokyo); Y. Uesaka, M. Kunimoto, S. Takeuchi, H. Kurono, Y. Shirota, Y. Koide, H. Ishiura, M. Yogo, K. Yamashita, K. Kaibara, T. Hasegawa, Y. Mikami, H. Yamakawa, Y. Yamada, H. Takeuchi (International Medical Center of Japan, Tokyo); T. Imasawa (National Hospital Organization Chiba-East National Hospital, Chiba); T. Nishiyama, Y. Kuroiwa, H. Kishida (Yokohama city University Graduate School of Medicine, Kanagawa); Y. Shiokawa, H. Kuroiwa, H. Kurita, H. Seyama, K. Nishiyama (Kyorin University School of Medicine, Tokyo); K. Hoya (Dokkyo Medical University Koshigaya Hospital, Saitama); T. Ueba, T. Haze, M. Sugiyama, (Kishiwada City Hospital, Osaka); T. Ishihiki, K. Kozuma, K. Kasawagi (Teikyo University School of Medicine, Tokyo); M. Yamasaki (Tokyo Women’s Medical University, Tokyo); S. Mocho, C. Toyoda (Daisan Hospital, The Jikei University School of Medicine, Tokyo); A. Hasegawa (Gunma University Graduate School of Medicine, Gunma); K. Satoh (Faculty of Medicine, University of Yamanashi, Yamanashi).

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