intolerant patients (61.8 %), and the refractory patients reached a response quicker than the intolerant patients (92.0 vs. 124 days, respectively). Both these phenomena could be related to the fact that the refractory patients received a higher median daily dose of anagrelide compared with the intolerant patients (2.622 vs. 1.695 mg/day). It is also interesting to note that up-titration of anagrelide was more pronounced in the refractory patients compared with the intolerant patients, thus possibly explaining the more rapid time to response in this patient group. However, no firm conclusions can be made due to the small numbers of patients in each of the subgroups.

The most frequently reported TEAEs in this study were anemia, headache, palpitations, and diarrhea. These are all commonly or very commonly reported anagrelide-related adverse reactions according to the European SPC [6] and US prescribing information [10], and are consistent with the results from the recent Phase I/II study in treatment-naïve Japanese patients with ET [9] and a post-marketing surveillance study in Korea [13]. There were two events of cerebral infarction, and one event of lacunar infarction on the study, which were possibly related to the thrombotic events that underlie the pathophysiological process of ET. The rate of withdrawal due to an AE observed in this study (15.1 %) is comparable to that reported in the PT-1 (27.2 %) and ANAHYDRET (5.7 %) trials [11, 12]; however, discontinuation definitions differ between studies.

In the present study, 45.3 % of patients entered the study with a history of anemia, and mean baseline levels of hemoglobin were relatively low (12.1 g/dL). This could be due to a number of factors including diet-related iron deficiency, which has been recognized in Japanese individuals [14], and it is also known that patients receiving long-term HC can experience anemia, due to its myelosuppressive effects [15]. TEAEs of anemia were reported in almost half [25/53 (47.2 %)] of the patients in the current study, but all cases were either considered mild or moderate in severity. Mean hemoglobin levels at the end of the study were 10.7 g/dL, representing a change from baseline of −1.4 g/dL. Anemia is a common side effect of anagrelide use (affecting 1–10 users in 100 [6]). Similar incidences of anemia have been reported in treatment-naïve Japanese patients with ET [5/12 (41.7 %)] [9]. However, in two previous, large-scale anagrelide studies in non-Japanese patients with ET, the incidence of anemia was reported at 9.0 % [11] and 7.9 % (iron-deficiency anemia and other anemia [12]). While the reasons for the high incidence of anemia reported in the current study are not fully understood, it can be hypothesized that several factors may have influenced this phenomenon, including the fact that no specific criteria for diagnosing anemia were included in the study protocol, and such AEs were only based on investigators' judgment.

Patients were treated in accordance with the European SPC for anagrelide [6] and received an initial starting dose of 1.0 mg/day for 1 week, divided into two separate doses of 0.5 mg. Doses were then titrated thereafter in order to find the clinically effective dose for each patient. In this study, the median daily dose was 1.90 mg/day (range 0.58–5.48 mg/day), which is within the European SPC [6] and the US prescribing information (for adult patients with thrombocythemia, secondary to myeloproliferative disorders) [10] recommended maintenance doses (1–3 and 1.5–3 mg/day, respectively). The median daily dose observed in this study is also similar to the dose reported in the recent Evaluation of Xagrid Efficacy and Long-term Safety (EXELS) study in Europe (1.5 mg/day) [16, 17]. The dosing observed in this present study further supports evidence from a previous study in treatment-naïve patients with ET [9] that Japanese-specific anagrelide dosing regimens are not required.

There was a broad range of anagrelide doses administered in the present study (0.5–7.0 mg/day). These are similar to those reported in the previous Japanese study in treatment-naïve patients with ET (0.5–5.5 mg/day) [9] and the EXELS European study (0.5–6.0 mg/day) [17]. These results indicate that individualized treatment regimens are required to ensure each patient receives the lowest effective dosage required to reduce and maintain platelet counts.

A limitation of the present study is that a comparator arm was not included because there was no approved second-line treatment for ET in Japan. This limits our ability to draw firm conclusions regarding our results; however, overall these data are in line with previously reported data from Caucasian patients and support the use of anagrelide in Japanese patients with ET. In addition, although a diagnosis of ET according to the WHO criteria was required for enrollment on the study, it was not reconfirmed at study entry, i.e. further bone biopsies were not carried out as it was considered too burdensome for the patient.

In conclusion, these data demonstrate that anagrelide effectively reduces platelet counts in high-risk Japanese patients with ET who are intolerant or refractory to their previous CRT, supporting the use of anagrelide as a second-line therapy for ET in this patient population. Dosing of anagrelide was comparable to previous studies of Caucasian patients, although it should be noted that individualized dosing is required to ensure patients achieve a maximum platelet response at the lowest effective dose. Anagrelide's safety profile in Japanese patients is consistent with the European SPC and US prescribing information.

Acknowledgments The study was funded by Shire Development LLC. Medical writing services were provided by Louise Prince, PhD, of iMed Comms and were funded by Shire. Editorial assistance in formatting, proofreading, copy editing, and fact checking was also provided by iMed Comms. iMed Comms was funded by Shire for support in writing and editing this manuscript. Shire also reviewed and edited the manuscript for scientific accuracy. Although Shire was involved in the analysis, interpretation, and fact checking of information, the content of this manuscript, the ultimate interpretation, and the decision to submit it for publication in the *International Journal of Hematology* was made by the authors independently. The authors thank the investigators who participated in this study.

Conflict of interest YK has provided consultancy for a clinical trial. YM has provided consultancy to Eisai and Kyowa Hakko Kirin, received payment for lectures including service on speakers' bureaus from GlaxoSmithKline, Kyowa Hakko Kirin, and Shire, and has a patent pending for a screening model of drugs for megakaryopoiesis. PW is an employee of, and holds stocks/stock options in, Shire Pharmaceuticals. JS is a former contractor of Shire Pharmaceuticals. HA is an employee of Shire Pharmaceuticals. SO has received honoraria from Pfizer, Bristol-Myers Squibb, and Novartis; he has also received donations from Kyowa Hakko Kirin and Chugai.

References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of tumours of haemopoietic and lymphoid tissues. 4th ed. Lyon: IARC Press; 2008.
2. Barbui T, Barosi G, Birgegard G, Cervantes F, Finazzi G, Griesshammer M, et al. Philadelphia-negative classical myeloproliferative neoplasms: critical concepts and management recommendations from European LeukemiaNet. J Clin Oncol. 2011;296:761–70.
3. Harrison CN, Bareford D, Butt N, Campbell P, Conneally E, Drummond M, et al. Guideline for investigation and management of adults and children presenting with a thrombocytosis. Br J Haematol. 2010;1493:352–75.
4. Dan K, Yamada T, Kimura Y, Usui N, Okamoto S, Sugihara T, et al. Clinical features of polycythemia vera and essential thrombocythemia in Japan: retrospective analysis of a nationwide survey by the Japanese Elderly Leukemia and Lymphoma Study Group. Int J Hematol. 2006;835:443–9.
5. Kiladjian JJ, Chevret S, Dosquet C, Chomienne C, Rain JD. Treatment of polycythemia vera with hydroxyurea and pipobroman: final results of a randomized trial initiated in 1980. J Clin Oncol. 2011;2929:3907–13.
6. European Medicines Agency. Xagrid summary of product characteristics, Shire Pharmaceuticals Ltd. Available from http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000480/WC500056557.pdf 2014 (Accessed on 5 Feb 2014).
7. Shire PLC. AGRYLIN (anagrelide hydrochloride). Available from www.shire.com/shireplc/en/products/other/AGRYLIN 2012 (Accessed on 28 Nov 2013).
8. Barosi G, Besses C, Birgegard G, Briere J, Cervantes F, Finazzi G, et al. A unified definition of clinical resistance/intolerance to hydroxyurea in essential thrombocythemia: results of a consensus process by an international working group. Leukemia. 2007;212:277–80.
9. Okamoto S, Miyakawa Y, Smith J, Hodgson I, Abhyankar B, Troy S, et al. Open-label, dose-titration and continuation study to assess efficacy, safety, and pharmacokinetics of anagrelide in treatment-naive Japanese patients with essential thrombocythemia. Int J Hematol. 2013;973:360–8.
10. US Food and Drug Administration (FDA). Summary of product characteristics. Available from http://www.accessdata.fda.gov/drugsatfda_docs/label/2004/20333s010lbl.pdf 2012 (Accessed on 16 Aug 2012).
11. Gisslinger H, Gotic M, Holowiecki J, Penka M, Thiele J, Kvasnicka HM, et al. Anagrelide compared to hydroxyurea in WHO-classified essential thrombocythemia: the ANAHYDRET Study, a randomized controlled trial. Blood. 2013;12110:1720–8.
12. Harrison CN, Campbell PJ, Buck G, Wheatley K, East CL, Bareford D, et al. Hydroxyurea compared with anagrelide in high-risk essential thrombocythemia. N Engl J Med. 2005;3531:33–45.
13. Nam S, Abhyankar B, Choi Y, Jung J. Safety of anagrelide in clinical practice: a multi-centre post-marketing surveillance study in Korea. Abstract at the 74th Annual Meeting of the Japanese Society of Hematology (JSH), Kyoto, Japan, 19–21 October 2012.
14. Shimomura T. Wakabayashi I [Regional differences in prevalence of anemia found by periodic health checkups at workplaces in Japan]. Sangyo Eiseigaku Zasshi. 2010;521:21–7.
15. Spivak JL, Hasselbalch H. Hydroxycarbamide: a user's guide for chronic myeloproliferative disorders. Expert Rev Anticancer Ther. 2011;113:403–14.
16. Gugliotta L, Besses C, Griesshammer M, Harrison C, Kiladjian JJ, Coll R, et al. Combination therapy of hydroxycarbamide with anagrelide in patients with essential thrombocythemia in the evaluation of Xagrid (R): efficacy and long-term safety study. Haematologica. 2014;99:679–87.
17. Besses C, Kiladjian JJ, Griesshammer M, Gugliotta L, Harrison C, Coll R, et al. Cytoreductive treatment patterns for essential thrombocythemia in Europe. Analysis of 3643 patients in the EXELS study. Leuk Res. 2013;372:162–8.

Int J Hematol
DOI 10.1007/s12185-015-1741-0

ORIGINAL ARTICLE

Clinical significance of IPF% or RP% measurement in distinguishing primary immune thrombocytopenia from aplastic thrombocytopenic disorders

Mikiko Sakuragi · Satoru Hayashi · Miho Maruyama · Osamu Kabutomori · Tomoko Kiyokawa · Keisuke Nagamine · Hisashi Kato · Hirokazu Kashiwagi · Yuzuru Kanakura · Yoshiaki Tomiyama

Received: 22 November 2014 / Revised: 6 January 2015 / Accepted: 15 January 2015

Abstract The diagnosis of primary immune thrombocytopenia (ITP) is based on differential diagnosis. Although the measurement of percentages of reticulated platelets (RP%) by flow cytometry is useful as a supportive diagnostic test, this method is nonetheless a time-consuming, laboratory-based assay. To identify alternative assays that are useful in daily practice, we compared three methods in parallel, IPF% measured by XE-2100 [IPF% (XE), Sysmex Corp.], IPF% measured by new XN-1000 [IPF% (XN)], and RP%. We examined 47 patients with primary ITP, 28 patients with aplastic thrombocytopenia (18 aplastic anemia and 10 chemotherapy-induced thrombocytopenia) and 80 healthy controls. In a selected experiment, we examined 16 patients with paroxysmal nocturnal hemoglobinuria (PNH) to examine the effect of hemolysis. As compared with IPF% (XE), IPF% (XN) showed better within-run reproducibility. The sensitivity and specificity for the diagnosis of ITP were 83.0 and 75.0 % for IPF% (XE), 85.1 and 89.3 % for IPF% (XN), and 93.6 and 89.3 % for RP%, respectively. Examination of PNH patients revealed that hemolysis and/or red blood cell fragments interfered with IPF% (XE) values, but not with IFP % (XN) values. Our results suggest that IPF% measured by XN-1000 may be of comparable value with RP% as a supportive diagnostic test for ITP.

Keywords Immune thrombocytopenia · Reticulated platelets · Differential diagnosis · Paroxysmal nocturnal hemoglobinuria · Thrombopoietin

M. Sakuragi · S. Hayashi · T. Kiyokawa · K. Nagamine · H. Kato · Y. Tomiyama (✉)
Department of Blood Transfusion, Osaka University Hospital, Suita, Osaka 565-0871, Japan
e-mail: yoshi@hp-blood.med.osaka-u.ac.jp

M. Maruyama · O. Kabutomori
Laboratory for Clinical Investigation, Osaka University Hospital, Suita, Osaka, Japan

H. Kato · H. Kashiwagi · Y. Kanakura · Y. Tomiyama
Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disease characterized by early platelet destruction due to anti-platelet autoantibodies and slightly impaired platelet production [1–3]. Despite recent advances in understanding of its pathophysiology, the diagnosis of ITP is still mainly based on differential diagnosis [4]. However, it is sometimes very difficult to distinguish ITP from isolated thrombocytopenia due to aplastic thrombocytopenic disorders such as aplastic anemia (AA) and amegakaryocytic thrombocytopenia. To resolve this issue, several laboratory-based assays have been developed: detection of anti-platelet autoantibodies, measurement of percentage of reticulated platelets (RP%) and plasma thrombopoietin (TPO) concentrations [3]. Regarding detection of platelet-associated autoantibodies, it has been shown that its specificity for the diagnosis of ITP is very high (80–90 %) in prospective studies. However, the drawback in this assay is its relatively low sensitivity as well as being time-consuming, laboratory-based assay: platelet-associated anti-GPIIb/IIIa and/or anti-GPIb/IX antibodies are detected in only 51–55 % of ITP [5–7]. Alternatively, measurement of RP% and plasma TPO concentrations is useful to distinguish between ITP and aplastic thrombocytopenic disorders [8–10]. RPs are reported to be younger platelets (i.e., immature platelets) that have been released recently into

Published online: 25 January 2015

the circulation and are probably analogous to reticulocytes reflecting erythropoiesis. RPs can be distinguished from mature platelets by their RNA contents using flow cytometry with an RNA-binding fluorochrome, such as thiazole orange, and RP% and absolute number of RPs are reflecting platelet production and hence platelet turnover [11, 12]. In ITP patients RP% was markedly increased compared with healthy controls, whereas RP% in patients with AA or chemotherapy-induced thrombocytopenia (CIT) was within normal range [10–12]. In contrast, plasma TPO levels in ITP patients are within normal range or only slightly increased, whereas those in patients with aplastic thrombocytopenic disorders are markedly increased [8–10]. Accordingly, Japanese ITP working group including us proposed preliminary diagnostic criteria for ITP by incorporating anti-platelet autoantibody detection, RP%, and plasma TPO level. In a multi-center prospective study, the criteria showed high sensitivity and specificity for the diagnosis of ITP [13]. However, the method for the measurement of RP% is nonetheless a time-consuming, laboratory-based assay and has not been standardized yet.

We have been seeking alternative assays to measure RP% that are useful in daily practice, although measurement of RP% by flow cytometry is the gold standard method. One candidate is measurement of percentage of immature platelet fraction (IPF%) using Sysmex XE-2100 (or XE-5000) automated hematology analyzer (Sysmex Corp., Kobe, Japan). This IPF% method becomes very popular because of its convenience [14]. However, we previously demonstrated that IPF% measured by XE-2100 showed less sensitivity and specificity as compared to RP% method to distinguish between ITP and AA patients [3, 15]. Thus, IPF% measured by XE-2100 was neither so accurate nor satisfactory in daily practice. To improve the accuracy of IPF% method, new generation analyzer, XN-1000 has been developed and become commercially available. In this study, we compared these three methods in parallel, IPF% measured by XE-2100 [IPF% (XE)], IPF% measured by XN-1000 [IPF% (XN)], and RP%, for their utility in differential diagnosis between ITP and aplastic thrombocytopenia (AA and CIT). In addition, effects of hemolysis in patients with paroxysmal nocturnal hemoglobinuria (PNH) on IPF% (XE), IPF% (XN), and RP% were examined.

Materials and methods

Subjects

For a period of 6 months (October 2013 through March 2014) we examined 47 patients with primary ITP [9 males and 38 females, age 59 ± 17 years, platelet count 57 ± 34 × 10^3/μl (mean ± SD)], 28 patients with aplastic (or hypoplastic) thrombocytopenia [18 AA and 10 chemotherapy-induced thrombocytopenia (CIT)] [11 males and 17 females, age 50 ± 15 years, platelet count 43 ± 28 × 10^3/μl (mean ± SD)], and 80 healthy controls [35 males and 45 females, age 34 ± 12 years, platelet count 269 ± 58 × 10^3/μl (mean ± SD)]. Diagnosis of primary ITP and aplastic anemia was based on reports from an international working group and International Agranulocytosis and Aplastic Anemia Study group, respectively [4, 16]. With regard to management of 47 patients with ITP, 19 patients managed with observation alone, 14 patients mainly with prednisolone, 9 patients with TPO receptor agonist (TPORA) and prednisolone, and 5 patients with TPORA only. Thus, 14 ITP patients treated with TPO receptor agonist such as eltrombopag and romiplostim were included, and 12 of these patients still showed thrombocytopenia less than 100 × 10^3/μl. Patients with CIT include 6 patients with AML, 2 patients with ALL, and 2 patients with MDS, and samples were obtained on day 1 or day 2 for myeloablative allogeneic hematopoietic stem cell transplantation. We obtained informed consent from all subjects, in accordance with the declaration of Helsinki. This study was approved by Osaka University Institutional Review Board.

In a selected experiment, we examined 16 patients with PNH to investigate effects of hemolysis and/or fragmentation of red blood cells (RBC) on the measurement of IPF% and RP%.

Measurement of RP%

RP% was measured as previously described with a slight modification [15]. In brief, 15-μl aliquots of whole blood anti-coagulated with ethylenediamineteraacetic acid (EDTA) were incubated with 5 μl of phycoerythrin-conjugated anti-CD42b monoclonal antibody (BD Pharmingen, Tokyo, Japan) and 20 μl of 2 % paraformaldehyde for 15 min at room temperature. After adding 1 ml of thiazole orange (Retic-COUNT; Becton–Dickinson, San Jose, CA, USA) diluted to 8 times by phosphate-buffered saline, the whole blood samples were centrifuged at 350 g for 30 s to remove red blood cells, and then the platelet-rich suspensions were incubated at room temperature for 90 min. RP% was analyzed on a flow cytometer (FACScan, Becton–Dickinson) by measuring 10,000 events in the CD42b-positive fraction. To exclude cell autofluorescence and instrument background, platelet-rich suspension without thiazole orange was prepared as a negative control for each sample.

Measurement of IPF% by Sysmex automated hematology analyzer XE-2100 and XN-1000

EDTA-anti-coagulated whole blood samples were also used to measure IPF% employing automated hematology

Table 1 Within-run reproducibility for measuring IPF% by XE-2100 and XN-1000

Sample	Platelet count ($10^3/\mu l$)	Number	IPF (%)	CV (%)
XE-2100				
Control-1	312.2 ± 7.2	10	2.29 ± 0.41	17.7
Control-2	339.8 ± 7.0	10	0.76 ± 0.13	16.9
Control-3	336.9 ± 6.8	10	1.76 ± 0.21	11.7
Control-4	256.8 ± 5.0	10	1.98 ± 0.21	10.6
ITP-1	37.8 ± 1.8	10	8.97 ± 1.26	14.0
ITP-2	24.7 ± 1.3	10	12.85 ± 1.83	14.3
XN-1000				
Control-1	293.5 ± 2.3	10	2.78 ± 0.07	2.7
Control-2	362.0 ± 2.8	10	0.56 ± 0.05	8.8
Control-3	314.9 ± 2.5	10	1.76 ± 0.09	5.2
Control-4	251.5 ± 2.4	10	2.68 ± 0.16	6.0
ITP-1	37.4 ± 0.5	10	11.84 ± 0.60	5.0
ITP-2	20.5 ± 0.8	10	13.40 ± 0.77	5.7

CV coefficient of variation

analyzer XE-2100 (XE, Sysmex) and the newer generation analyzer, XN-1000 (XN, Sysmex). XE used polymethine and oxazine to stain nucleic acid and RET-channel for the measurement of IPF, whereas XN used only oxazine and PLT-F channel to more accurately detect platelets and IPF [17]. All samples were measured within 7 h of venesection.

Measurement of plasma TPO concentrations

Plasma TPO concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit as previously described (R & D Systems, Minneapolis, MN, USA) [10].

Statistical analysis

The differences between mean values were evaluated using Student t test and a p value less than 0.05 was considered statistically significant. We analyzed sensitivity and specificity of IPF% (XE), IPF% (XN), and RP% for the diagnosis of ITP as previously described [10]. Constructed receiver operating characteristics (ROC) curves were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

Within-run reproducibility

We first examined within-run reproducibility for IPF% measurement in 4 control subjects and 2 ITP patients employing XE and XN in parallel. Each sample was measured 10 times, and mean intra-assay coefficients of variation [CV(%)] for these samples were 14.2 ± 2.8 and 5.6 ± 2.0 % for XE and XN, respectively ($p < 0.001$) (Table 1).

IPF%, RP%, and plasma TPO levels in thrombocytopenic disorders due to either accelerated platelet destruction or deficient platelet production.

We examined 47 patients with ITP as a thrombocytopenic disorder due to early platelet destruction and 18 patients with aplastic anemia and 10 patient with CIT as aplastic (or hypoplastic) thrombocytopenic disorders. IPF% values obtained from 80 control subjects were 2.5 ± 1.3 and 2.2 ± 1.2 % (mean ± SD) for XE and XN, respectively. As we defined an upper limit for healthy control subjects as mean + 3SD in this study, the upper limits were 6.4 and 5.8 % for XE and XN, respectively. RP% value obtained from 80 control subjects measured by flow cytometry was 4.8 ± 1.1 % (mean ± SD) and its upper limit of reference range was defined as 8.1 % (mean + 3SD).

Figure 1 shows correlations between IPF% (XE), IPF% (XN), and RP%. Good linear correlation between IPF% (XE) and IPF% (XN) was obtained ($r = 0.94$), whereas only moderate correlation between RP% and IPF% (XE) ($r = 0.72$) or IPF% (XN) ($r = 0.71$) was obtained. IPF and RPs were measured with oxazine and thiazole orange, respectively, and they were thought to be equivalent. However, our data suggested that IPF and RPs were similar, but not quantitatively identical. Next, we examined the sensitivity and specificity of IPF% and RP% to distinguish ITP from AA/CIT. There was no significant difference in platelets count between two groups (57 ± 34 × $10^3/\mu l$ for ITP, 43 ± 28 × $10^3/\mu l$ for AA/CIT). Figure 2 shows IPF% (XE), IPF% (XN) and RP% in ITP patients and AA/CIT patients. As expected, IPF% (XE), IPF% (XN) and RP% showed clear difference between ITP and AA/CIT. However, elevated values were detected in 83.0, 85.1, and 93.6 % of ITP measured by IPF% (XE), IPF% (XN) and

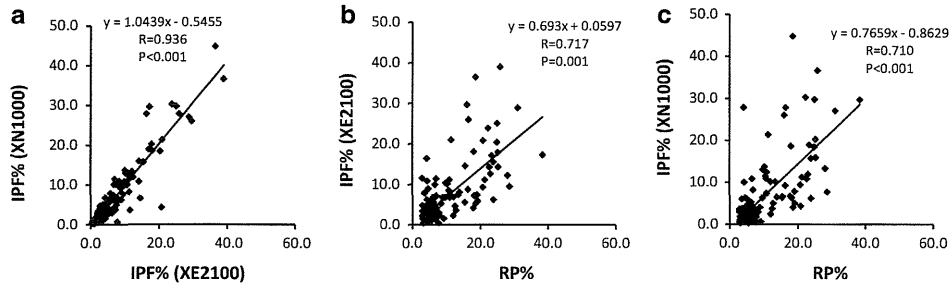

Fig. 1 Correlations between IPF% (XE-2100), IPF% (XN-1000), and RP% by flow cytometry. **a** IPF% (XE-2100) versus IPF% (XN-1000), **b** RP% versus IPF% (XE-2100), and **c** RP% versus IPF% (XN-1000)

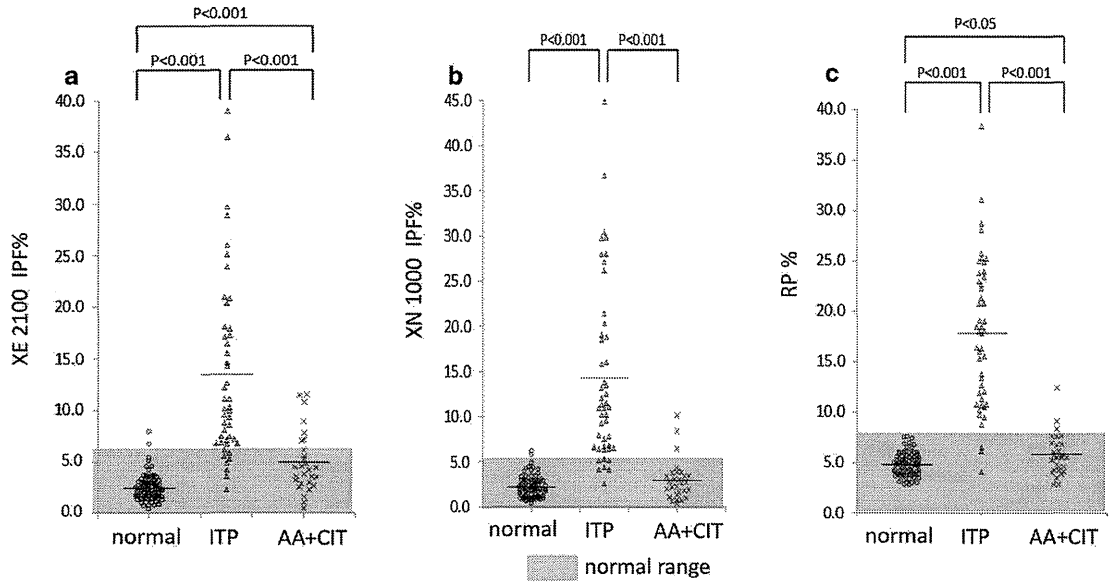

Fig. 2 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in healthy controls, patients with primary immune thrombocytopenia (ITP), and patients with aplastic anemia (AA) or chemotherapy-induced thrombocytopenia (CIT). *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy controls)

RP%, respectively. In sharp contrast, the elevated values were detected in 25.0, 10.7, and 10.7 % of AA/CIT by IPF% (XE), IPF% (XN) and RP%, respectively (Fig. 2). As compared with IPF% (XN) and RP%, IPF% measured by XE-2100 appeared to be less frequently elevated in ITP and much frequently elevated in AA/CIT. Thus, the sensitivity and specificity of the elevation of IPF% or RP% for the diagnosis of ITP were 83.0 and 75.0 % for IPF% (XE), 85.1 and 89.3 % for IPF% (XN), and 93.6 and 89.3 % for RP%, respectively (Table 2).

In parallel we measured plasma TPO concentration in each sample, which further confirmed the diagnosis (Fig. 3). TPO levels obtained from 80 control subjects were 16.3 ± 21.8 pg/ml (mean ± SD), and the upper limit of reference range for TPO was 81.7 pg/ml (mean + 3SD). Patients with AA/CIT showed markedly increased plasma TPO levels, whereas patients with ITP showed normal or modestly increased TPO levels. In addition, we measured mean platelet volume (MPV) for ITP and AA/CIT. However, MPV could be measured by XN-1000 in only 28 out of 47 ITP patients, probably because of abnormal size distribution of platelet volume in ITP. Nonetheless, MPVs for ITP (12.5 ± 1.0 fl, $n = 28$) were significantly larger than 80 controls (10.5 ± 1.0 fl, $p < 0.001$) and 23 AA/CIT (10.7 ± 0.9 fl, $p < 0.001$).

Receiver operating characteristic (ROC) analysis

ROC curves were constructed for the sensitivity and specificity of the differential diagnosis of ITP from AA/CIT patients, and IPF% (XE), IPF% (XN), and RP% data were analyzed separately. ROC curve for IPF% (XE), IPF%

Table 2 Sensitivity, specificity, and predictive values of IPF% (XE), IPF% (XN), and RP% for the diagnosis of ITP

	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
Upper panel includes ITP patients treated with TPORA (total 47 ITP patients)				
IPF% (XE-2100)	83.0	75.0	84.8	72.4
IPF% (XN-1000)	85.1	89.3	93.0	78.1
RP%	93.6	89.3	93.6	89.3
Lower panel excludes ITP patients treated with TPORA (total 33 ITP patients)				
IPF% (XE-2100)	78.8	75.0	78.8	75.0
IPF% (XN-1000)	81.8	89.3	90.0	80.6
RP%	90.9	89.3	90.9	89.3

(XN), and RP% showed area under curve (AUC) of 0.863, 0.956, and 0.959, respectively (Fig. 4).

IPF% and RP% in patients with PNH

To examine effects of hemolysis on the measurement of IPF% and RP% we examined patients with PNH. Ten patients out of 16 PNH patients were treated with eculizumab, a humanized monoclonal antibody against terminal complement protein C5 that inhibits terminal complement activation. As shown in Fig. 5, 6 PNH patients showed elevated IPF% (XE), whereas none and only two patients showed elevated IPF% (XN) and RP%, respectively. Five out of 6 PNH patients with elevated IPF% (XE) were treated with eculizumab, suggesting active hemolysis may interfere with the measurement of IPF% by XE-2100.

Discussion

Recent in vivo vital imaging as well as biochemical and genetic approaches have revealed the mechanism of platelet production (thrombopoiesis) from mature megakaryocytes. Mature megakaryocytes localized in bone marrow sinusoids extend proplatelets into the lumen of the sinusoids, and then new platelets are shed as fragments from the tips of intravascular proplatelets [18, 19], and newly produced platelets can be distinguished from mature platelets by their content of RNA as RPs by flow cytometry [20]. Despite our progress in understanding of pathophysiology of ITP as well as mechanism of thrombopoiesis, diagnosis of ITP has been still based on differential diagnosis [1–3].

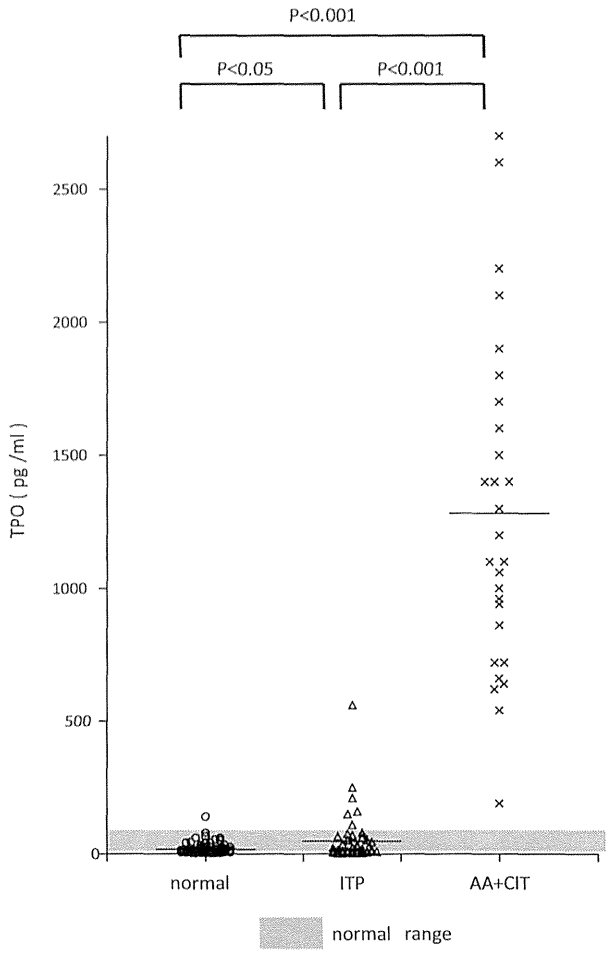

Fig. 3 Plasma thrombopoietin (TPO) levels in healthy controls, patients with ITP, and patients with AA/CIT. Patients with AA/CIT showed markedly increased plasma TPO levels, whereas patients with ITP showed normal or modestly increased TPO levels

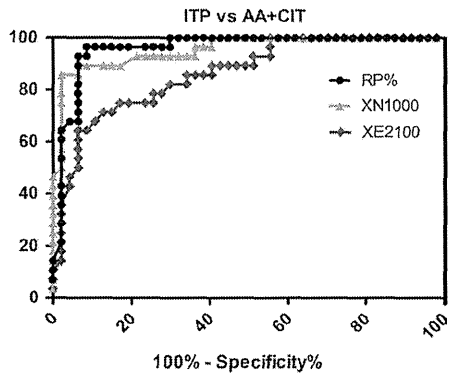

Fig. 4 Receiver operating characteristic (ROC) analysis. ROC curves were constructed for the sensitivity and specificity of the differential diagnosis of ITP from AA/CIT patients. ROC curve for IPF% (XE-2100), IPF% (XN-1000), and RP% showed area under curve (AUC) of 0.863, 0.956, and 0.959, respectively

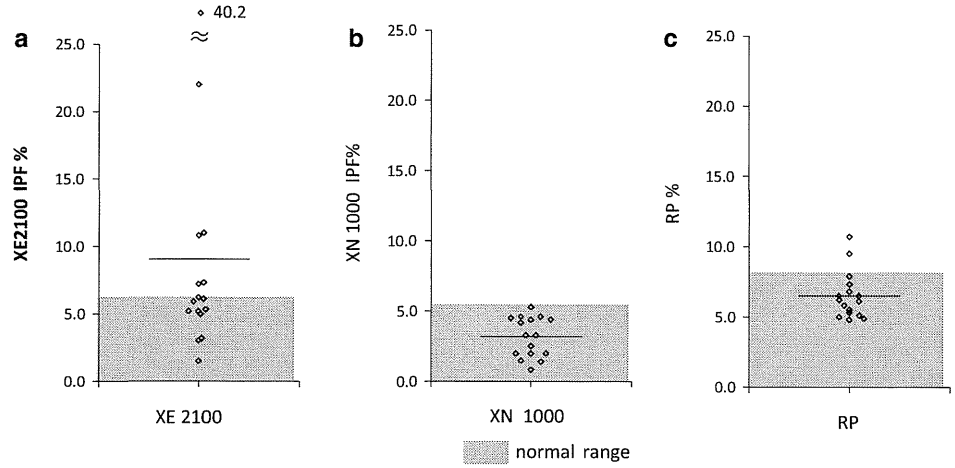

Fig. 5 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in patients with PNH. *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy controls)

Several laboratory-based tests, detection of platelet-associated autoantibodies, measurement of RP% and plasma TPO level, could be useful for the diagnosis of ITP [10]. However, the measurement of RP% is laboratory-based assay, and not used in daily practice yet. In addition, methods for RP% measurement have not been standardized. High concentrations of thiazole orange as well as longer incubation time, more than 2.5 h, induced dramatically higher fluorescence intensities, probably due to penetration of the dye into the dense granules [20]. Accordingly, we used 8-times-diluted thiazole orange and shorter incubation time (90 min) to measure RP%. To examine the reliability of automated hematology analyzer-based IPF% method for the differential diagnosis of ITP, we compared IPF% (XE), IPF% (XN), and RP% in parallel. Platelets were precisely monitored by anti-CD42b antibody and 10,000 events were analyzed even under thrombocytopenic conditions in the RP% method, but not in IPF% (XE) or IPF% (XN). However, in the new XN series a novel PLT-F channel was introduced to more specifically gate platelets than in XE series [17, 20]. Actually, we confirmed that data obtained by XN-1000 were much more accurate than XE-2100 regarding within-run reproducibility.

We examined the correlation between IPF% (XE), IPF% (XN), and RP%. Excellent correlation between IPF% (XE) and IPF% (XN) was obtained, while only moderate correlation even between RP% and IPF% (XN) existed. The difference between IPF% and RP% is probably caused by the difference in the fluorescent dyes: oxazine and thiazole orange. Our data first revealed that IFP % (XN) values and RP% values were moderately related, but not quantitatively identical.

In a selected experiment we examined samples obtained from PNH patients, since in XE-2100 RBC fraction and platelet fraction were relatively close each other and both polymethine and oxazine were used as fluorescent dyes. As expected, hemolysis and/or RBC fragments interfered with IPF% (XE) values and 6 out of 16 PNH samples showed elevated IPF% (XE) values. However, none and two samples showed elevated IFP % (XN) and RP%, respectively. In contrast to XE series employing both polymethine and oxazine as fluorescent dyes to measure reticulocytes and immature platelets, XN-1000 employs only oxazine to measure immature platelets more specifically. In addition, the novel PLT-F channel enables us to more accurately differentiate platelets from other cells and interfering particles such as RBC fragments than XE series [21]. The false positive results in IPF% (XE) may be partially caused by non-specific binding of polymethine to RBC fragments. These data suggested that influence of hemolysis and/or RBC fragmentation was only minimal on measurement of IPF% by XN-1000 as well as RP% by flow cytometry.

We then compared the sensitivity and specificity of IPF% (XE), IPF% (XN), and RP% for the differential diagnosis between ITP and AA/CIT. There were clear differences in plasma TPO levels between ITP and AA/CIT. We confirmed our previous data that IPF% (XE) showed less sensitivity and specificity (83.0 and 78.6 %, respectively) as compared with RP% (93.6 and 89.3 %, respectively) [15]. In sharp contrast, IPF% by XN-1000 showed comparable sensitivity and specificity (85.1 and 92.9 %, respectively) with RP%. The sensitivity and specificity of IPF% (XE) was relatively high as compared with our previous study (sensitivity 67 %, specificity 63 %). This difference is probably due to the inclusion of ITP patients treated with TPORA in this study, because TPORA effectively increased the absolute number of RPs (and IPF) and maintained elevated RP% (and IPF%) even after improvement

of thrombocytopenia [14]. In fact, exclusion of 14 ITP patients treated with TPORA led to the decrease in the sensitivity of these assays [IPF% (XE) 78.8 %, IPF% (XN) 81.8 %, and RP% 90.9 %] (Table 2). ROC curve for IPF% (XE), IPF% (XN), and RP% showed area under curve (AUC) of 0.86, 0.96, and 0.96, respectively, indicating that IPF% measured by XN-1000 may be comparable with RP% by flow cytometry.

In summary, the data obtained from our study suggested that IPF% measured by XN-1000 may be of comparable value with RP% as a supportive diagnostic test in distinguishing between thrombocytopenic disorders due to early platelet destruction such as ITP and aplastic thrombocytopenic disorders such as aplastic anemia. In addition, automated hematology analyzer is easy to handle and suitable for daily practice. Limitation of our study is that we examined patients with definitive diagnosis from single institute. Multi-center prospective study would be necessary to further confirm our data.

Acknowledgments This work was supported by Grant-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan and the Ministry of Health, Labor and Welfare in Japan.

Conflict of interest Automated hematology analyzers, XE-2100 and XN-1000, were supplied by Sysmex Corp. During this study the authors have no other COI to declare.

References

1. Cines DB, Blanchette VS. Immune thrombocytopenic purpura. N Engl J Med. 2002;346:995–1008.
2. McMillan R. The pathogenesis of chronic immune thrombocytopenic purpura. Semin Hematol. 2007;44:S3–11.
3. Kashiwagi H, Tomiyama Y. Pathophysiology and management of primary immune thrombocytopenia. Int J Hematol. 2013;98:24–33.
4. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold 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.
5. Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH. Prospective evaluation of the clinical usefulness of an antigen specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. Blood. 1996;88:194–201.
6. McMillan R, Wang L, Tani P. Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP). J Thromb Haemost. 2003;1:485–91.
7. Tomiyama Y, Kosugi S. Autoantigenic epitopes on platelet glycoproteins. Int J Hematol. 2005;81:100–5.
8. Kosugi S, Kurata Y, Tomiyama Y, Tahara T, Kato T, Tadokoro S, et al. Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. Br J Haematol. 1996;93:704–6.
9. Emmons RV, Reid DM, Cohen RL, Meng G, Young NS, Dunbar CE, et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood. 1996;87:4068–71.
10. Kurata Y, Hayashi S, Kiyoi T, Kosugi S, Kashiwagi H, Honda S, et al. Diagnostic value of tests for reticulated platelets, plasma glycocalicin, and thrombopoietin levels for discriminating between hyperdestructive and hypoplastic thrombocytopenia. Am J Clin Pathol. 2001;115:656–64.
11. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood. 1990;75:116–21.
12. Richards EM, Baglin TP. Quantitation of reticulated platelets: methodology and clinical application. Br J Haematol. 1995;91:445–51.
13. Kuwana M, Kurata Y, Fujimura K, Fujisawa K, Wada H, Nagasawa T, et al. Preliminary laboratory based diagnostic criteria for immune thrombocytopenic purpura: evaluation by multicenter prospective study. J Thromb Haemost. 2006;4:1936–43.
14. Barsam SJ, Psaila B, Forestier M, Page LK, Sloane PA, Geyer JT, et al. Platelet production and platelet destruction: assessing mechanisms of treatment effect in immune thrombocytopenia. Blood. 2011;117:5723–32.
15. Hayashi S, Nishiyama M, Suehisa E, Kashiwagi H, Kurata Y, Tomiyama Y. Comparison between two methods for the measurement reticulated platelet and their clinical significance—flow cytometry (FCM) method and IPF method using automated hematology analyzer (XE-2000). Rinsho Byori. 2009;57:1039–44.
16. International agranulocytosis and aplastic anemia study. Incidence of aplastic anemia: the relevance of diagnostic criteria. By the International Agranulocytosis and Aplastic Anemia Study. Blood. 1987;70:1718–21.
17. van der Linden N, Klinkenberg LJ, Meex SJ, Beckers EA, de Wit NC, Prinzen L. Immature platelet fraction measured on the Sysmex XN hemocytometer predicts thrombopoietic recovery after autologous stem cell transplantation. Eur J Haematol. 2014;93:150–6.
18. Junt T, Schulze H, Chen Z, Massberg S, Goerge T, Krueger A, et al. Dynamic visualization of thrombopoiesis within bone marrow. Science. 2007;317:1767–70.
19. Zhang L, Orban M, Lorenz M, Barocke V, Braun D, Urtz N, et al. A novel role of sphingosine 1-phosphate receptor S1pr1 in mouse thrombopoiesis. J Exp Med. 2012;209:2165–81.
20. Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood. 1990;75:116–21.
21. Tanaka Y, Tanaka Y, Gondo K, Maruki Y, Kondo T, Asai S, et al. Performance evaluation of platelet counting by novel fluorescent dye staining in the XN-series automated hematology analyzers. J Clin Lab Anal. 2014;28:341–8.

ELSEVIER

Experimental Hematology

Experimental Hematology 2015;■:■–■

Quantitative polymerase chain reaction analysis with allele-specific oligonucleotide primers for individual IgH VDJ regions to evaluate tumor burden in myeloma patients

Q11 Hiroshi Sata[a], Hirohiko Shibayama[a], Ikuhiro Maeda[b], Yoko Habuchi[a], Eiji Nakatani[c], Kentaro Fukushima[a], Jiro Fujita[a], Sachiko Ezoe[a], Seiji Tadokoro[a], Tetsuo Maeda[a], Masao Mizuki[a], Satoru Kosugi[d], Masashi Nakagawa[e], Shuji Ueda[f], Masato Iida[g], Yukihiro Tokumine[h], Yasuhiko Azenishi[i], Hideki Mitsui[j], Kenji Oritani[a], and Yuzuru Kanakura[a]

Q1 [a]*Department of Hematology and Oncology, Osaka University Graduate School of Medicine;* [b]*Central Laboratory for Clinical Investigation, Osaka University Hospital;* [c]*Department of Statistical Analysis, Translational Research Informatics Center;* [d]*Department of Internal Medicine, Toyonaka Municipal Hospital;* [e]*Department of Hematology and Oncology, Nissay Hospital;* [f]*Department of Internal Medicine, Hyogo Prefectural Nishinomiya Hospital;* [g]*Department of Hematology, Kawasaki Hospital;* [h]*Department of Hematology, Itami City Hospital;* [i]*Department of Internal Medicine, Minoh City Hospital;* [j]*Department of Hematology, Otemae Hospital*

(Received 3 September 2014; revised 2 December 2014; accepted 5 January 2015)

Quantitative polymerase chain reaction (PCR) with patient-specific, allele-specific oligonucleotide (ASO) primers for individual immunoglobulin H VDJ region (ASO-PCR) amplification was performed using several sources of clinical material, including mRNA from peripheral blood cells (PBMNCs), whole bone marrow cells (BMMNCs), and the $CD20^+CD38^-$ B-cell population in bone marrow, as well as cell-free DNA from the sera of patients with multiple myeloma (MM). We designed the ASO primers and produced sufficient PCR fragments to evaluate tumor burden in 20 of 30 bone marrow samples at diagnosis. Polymerase chain reaction amplification efficiency depended on primer sequences because the production of ASO-PCR fragments did not correlate with serum M-protein levels. However, the ASO-PCR levels in BMMNCs showed statistically significant correlations with those in PBMNCs and $CD20^+CD38^-$ B-cells. The good association between the BMMNC and PBMNC data indicated that PBMNCs could be a suitable source for monitoring minimal residual disease (MRD). In the case of cell-free DNA, ASO-PCR levels showed a unique pattern and remained high even after treatment. Because the sequence information for each ASO-PCR product was identical to the original, the cell-free DNA might also be useful for evaluating MRD. Moreover, the ASO-PCR products were clearly detected in 17 of 22 mRNA samples from $CD20^+CD38^-$ populations, suggesting that MM clones might exist in relatively earlier stages of B cells than in plasma cells. Thus, ASO-PCR analysis using various clinical materials is useful for detecting MRD in MM patients as well as for clarifying MM pathogenesis.

Multiple myeloma (MM) is a B-cell malignancy characterized by the monoclonal expansion of plasma cells in bone marrow (BM) and the secretion of paraprotein in the serum and/or urine. Because high-dose chemotherapy with auto–hematopoietic stem cell transplantation, along with novel therapeutic agents, such as proteasome inhibitors and immunomodulatory drugs, increases response rates and extent of responses, the prognosis for MM patients has improved dramatically [1,2]. Nonetheless, most patients eventually relapse or develop progressive phases, which suggests the survival of malignant cells with proliferative capacity even after administration of those powerful regimens [3]. Improving treatment response and survival for MM patients requires evaluating and monitoring minimal residual disease (MRD) during treatment [4,5]. Among methods for MRD evaluation in MM patients, polymerase

Offprint requests to: Dr. Hirohiko Shibayama, Osaka University Graduate School of Medicine, Department of Hematology and Oncology, 2-2, Yamada-oka, Suita, Osaka 565-0871, Japan.; E-mail: hiro@bldon.med.osaka-u.ac.jp

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2015.01.002.

0301-472X/Copyright © 2015 ISEH - International Society for Experimental Hematology. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.exphem.2015.01.002

chain reaction (PCR) with patient-specific allele-specific oligonucleotide (ASO) primers for individual immunoglobulin H (IgH) VDJ regions (ASO-PCR) is considered the most sensitive [6,7]. Although previous reports have described measuring MRD with ASO-PCR, they usually involved analysis of BM cells by qualitative or semiquantitative methods [8,9].

In this study, we quantified ASO-PCR products using peripheral blood mononuclear cells (PBMNCs) as well as BM mononuclear cells (BMMNCs). In addition, we quantified ASO-PCR products for mRNA from $CD20^{+}38^{-}$ B-cells in BM to examine whether clonogenic cells are present in a relatively earlier B-cell fraction; we also quantified cell-free DNA from the sera to examine whether DNA fragments from MM cells are present in peripheral blood.

Materials and methods

Patients and samples

This study was performed according to the guidelines of the ethical committee of Osaka University Hospital and was approved by the Institutional Review Board of Osaka University Hospital and 19 related hospitals (HANDAI Clinical Blood Club). We analyzed 30 MM cases registered from December, 2011, until November, 2012. All patients gave written, informed consent for the molecular analysis. Remission status and disease progression were defined according to the International Myeloma Working Group criteria [10], with the only exception being that complete remission (CR) was not confirmed by BM biopsy. Fluorescence in situ hybridization analysis of t(4;14), t(11;14), t(14;16), and del(17p13) for BM-derived interphase cells was performed.

Design of a sense primer for detection of a specific immunoglobulin H VDJ region

The mononuclear cells were separated from the BM of MM patients at diagnosis. The $CD38^{high}CD20^{-}$ BMMNCs were sorted by using a FACS Aria cell sorter (Becton Dickinson, Heidelberg, Germany). Genomic DNA was extracted from the sorted $CD38^{high}CD20^{-}$ BMMNCs using the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The IgH VDJ regions were amplified by PCR with primers from the VH family of primers for sense and the JH consensus primer (5′-CTTACCT GAGGAGACGGTGACC) for antisense [11]. The amplified PCR fragments were sequenced. Based on each sequence, each patient's ASO primers were individually designed according to the methods described by van der Velden et al. [11].

Preparation of standard samples for real-time polymerase chain reaction

DNA from $CD38^{high}CD20^{-}$ BMMNCs of each patient was amplified using each specific IgH VDJ region primer and a consensus probe corresponding to the JH region. Subsequently, the PCR products were incorporated into the PCR 2.1-TOPO vector (TOPO TA Cloning Kit, Life Technologies, Grand Island, NY), and sequence analysis was carried out to confirm whether the insert carried the target sequence. These plasmids were used as templates to draw each standard curve.

Extraction of mRNA from bone marrow and peripheral blood mononuclear cells and $CD20^{+}CD38^{-}$ B-cells and of cell-free DNA from patient sera

The mRNA was extracted from MM patient BMMNCs, PBMNCs, and $CD20^{+}38^{-}$ B-cells using the QIAGEN RNeasy Mini Kit (QIAGEN) at diagnosis, as well as at the follow-up time points (6 months [6mo] and 12 months [12mo] after the start of the treatment). DNA was extracted from the sera of the patients at the same time using the WAKO DNA Extractor SP kit (Wako Pure Chemical Industries, Osaka, Japan).

Real-time quantitative polymerase chain reaction of the IgH gene

After mRNA was reversed transcribed into cDNA using M-MLV Reverse Transcriptase (Life Technologies), real-time reverse transcription PCR (RT-PCR) was performed on a Takara PCR Thermal Cycler Dice (Takara, Shiga, Japan) according to the protocol described below. The pairs of primers used for the quantification of IgH mRNA were *MM-MRD-5′* (0.3 μmol/L; sequences in Supplementary Table E1, online only, available at www.exphem.org) and *JH-3′* (0.3 μmol/L; 5′-CTTACCTGAGGAGACGGT GACC-3′). The primer pairs for the quantification of β-actin mRNA as an internal control were *ACF* (0.3 μmol/L; 5′-TGGA CATCCGCAAAGACCTG-3′) and *ACR* (0.3 μmol/L; 5′- AAG TACTCCGTGTGGATCGG-3′). The reaction mix for real-time RT-PCR was the SYBR Premix Dimer Eraser (Takara). The conditions for real-time RT-PCR were as follows: 95°C for 30 sec, 40 cycles of 95°C for 5 sec, 60°C for 30 sec, and 72°C for 30 sec. Values for IgH mRNA/β-actin mRNA (IgH/β-actin) were considered to indicate the real-time RT-PCR product levels (ASO-PCR levels). We confirmed that the sequences of the ASO-PCR products of interest from BMMNCs, PBMNCs, $CD20^{+}CD38^{-}$ B-cells, and cell-free DNA were identical to the originally designed versions.

Intra-assay precision of quantitative real-time reverse transcription polymerase chain reaction

Intra-assay precision of real-time RT-PCR was evaluated with 10 replicates of the plasmid. Plasmid was subjected to seven phases of dilution, from 9.81×10^{6} copies to 9.81×10^{0} copies, then measured 10 times each. For example, in the case of patient #8, the variation coefficients for the Ct values were 1.06% for 9.81×10^{6} copies, 1.01% for 9.81×10^{5} copies, 0.66% for 9.81×10^{4} copies, 0.89% for 9.81×10^{3} copies, 0.86% for 9.81×10^{2} copies, 1.12% for 9.81×10^{1} copies, and 2.09% for 9.81×10^{0} copies.

Limit of detection of quantitative real-time reverse transcription polymerase chain reaction

The smallest plasmid copy number was measured 10 times, and the minimal copy number detected by real-time RT-PCR was calculated by Ct applied to a standard curve. The resulting sensitivity was 9.81 copies.

Statistical analysis

To examine the correlations of the IgH/β-actin levels in BMMNCs with the ASO-PCR products in various clinical materials, as well as M-protein and percent of BM plasma cells, we performed univariate regression analysis and calculated the corresponding Spearman's correlation. An effect was always considered to be

Table 1. Patient characteristics

	n = 30
Median age (range)	64.5 (36–83)
Sex	
M	16 (53.3%)
F	14 (46.7%)
Type	
IgG	18 (60%)
IgA	7 (23.3%)
BJP	5 (16.7%)
ISS	
I	7 (23.3%)
II	9 (30%)
III	14 (46.7%)
Abnormal cytogenetics	
t(4;14)	4 (13.3%)
t(11;14)	4 (13.3%)
t(14;16)	1 (3.3%)
del(17p13)	2 (6.7%)
Best response (n = 23)	
CR	4 (17.4%)
VGPR	9 (39.1%)
PR	6 (26.1%)
SD	4 (17.4%)

ISS = International Staging System; *SD* = stable disease.

statistically significant if the p value of its corresponding statistical test was smaller than 0.05. For the statistical analysis, R version 3.0.2 was used.

Results

Patient characteristics

A summary of patient characteristics is given in Table 1, and all patient data are shown in Supplementary Table E2 (online only, available at www.exphem.org). Thirty patients with MM (7 with stage I, 9 with stage II, and 14 with stage III, according to the International Staging System [10]) were enrolled in this study. The median age in this cohort was 64.5 years (range = 36–83); the ratio of men to women was 16:14; and the numbers of IgG-, IgA-, and Bence Jones protein (BJP)-type patients were 18, 7, and 5, respectively (Table 1). The status of cytogenetic abnormality by fluorescent in situ hybridization was established in 4 for t(4;14), 4 for t(11;14), 1 for t(14;16), and 2 for del(17p13) (Table 1). Of 23 patients who were fully followed up, 4 reached CR, 9 achieved very good partial response (VGPR), 6 showed partial response (PR), and 4 remained in stable disease during the first year after the start of the treatment (Table 1).

Comparison of the IgH/β-actin values in between bone marrow and peripheral blood mononuclear cells

The values for IgH/β-actin (ASO-PCR values) in BMMNCs varied from 10^{-9} to 10^{2} at diagnosis (Fig. 1A); therefore, the efficiency of amplification by ASO-PCR seemed to depend on the designed primer sequences. This possibility was in part supported by the fact that the ASO-PCR levels

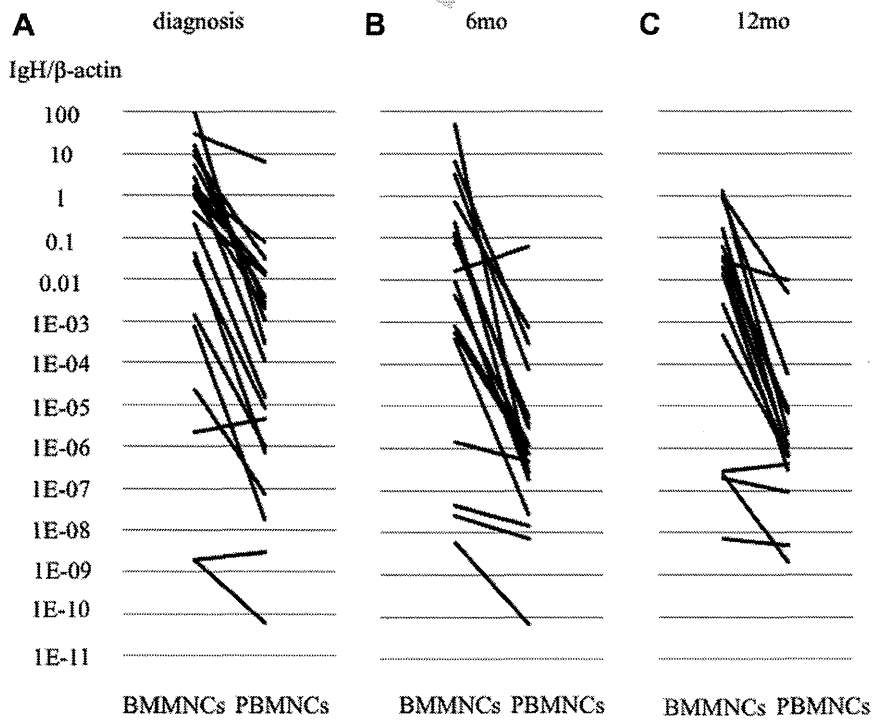

Figure 1. Comparison of IgH/β-actin values in between BMMNCs and PBMNCs. IgH/β-actin values in both BMMNCs and PBMNCs from each patient at diagnosis, at 6mo, and at 12mo are shown. Total RNA was prepared from BMMNCs and PBMNCs at (**A**) diagnosis, (**B**) 6mo, and (**C**) 12mo, and subjected to ASO-PCR with patient-specific designed primers. We also measured β-actin mRNA levels for each sample, and the IgH/β-actin values were considered to indicate ASO-PCR product levels.

Table 2. Correlation of the IgH/β-actin levels in BMMNCs with several biomarkers at diagnosis, 6mo, and 12mo

Variable (reference or unit)	Coefficient	95% CI	*p* value
Diagnosis			
IgH/β-actin (PBMNCs)	0.98	0.69–1.26	<0.001
IgH/DNA (cell-free DNA)	0.81	−0.10–1.71	0.078
IgH/β-actin ($CD20^+CD38^-$)	1.35	0.81–1.89	<0.001
Serum IgG	0.56	−2.99–4.10	0.740
Serum IgA	−4.37	−19.15–10.41	0.458
% plasma cells	−2.06	−6.13–2.02	0.304
6mo			
IgH/β-actin (PBMNCs)	0.83	0.15–1.51	0.020
IgH/DNA (cell-free DNA)	0.76	−0.36–1.89	0.169
Serum IgG	1.47	−1.34–4.27	0.280
Serum IgA	−1.03	−16.43–14.37	0.845
% plasma cells	1.32	−0.28–2.91	0.100
12mo			
IgH/β-actin (PBMNCs)	1.90	0.96–2.84	0.001
IgH/DNA (cell-free DNA)	−0.05	−1.53–1.43	0.942
Serum IgG	2.75	−0.34–5.83	0.076
Serum IgA	9.93	−4.71–24.58	0.120
% plasma cells	2.15	−3.77–8.08	0.427

CI = Confidence interval.
All *p* values obtained by univariate regression analysis (R version 3.0.2).

in BMMNCs correlated with those in PBMNCs, but not to the percent of plasma cells in BM or the values for M-protein (Table 2).

The ASO-PCR levels in PBMNCs from most of the patients rapidly decreased after treatment. At all time points, the ASO-PCR levels in PBMNCs were lower than those in BMMNCs (Fig. 1; Supplementary Table E2; online only, available at www.exphem.org).

The IgH/β-actin levels in PBMNCs showed a statistically significant correlation with those in BMMNCs at diagnosis (Spearman's $\rho = 0.98$, $p < 0.001$; Table 2; Fig. 2), 6mo (Spearman's $\rho = 0.83$, $p = 0.020$; Table 2), and 12mo (Spearman's $\rho = 1.90$, $p = 0.001$; Table 2). Therefore, ASO-PCR using PBMNCs as well as BMMNCs is likely to be suitable for MRD evaluation. The ASO-PCR levels in BMMNCs did not correlate with either serum M protein (IgG and IgA) levels or the percent of plasma cells in BM (Fig. 2; Table 2).

Comparing the kinetics of IgH/β-actin levels in BMMNCs and PBMNCs and M-protein levels, Figure 3 shows those in representative patients achieving VGPR or CR whose ASO-PCR values were more than 10^{-8} at each time point and more than 10^{-4} at diagnosis. It is noteworthy that the IgH/β-actin levels were decreased after treatment and reflected tumor burden well individually. In addition, the ASO-PCR products from BMMNC samples could be evaluated even when M-protein was not detected. The changes of ASO-PCR levels were more than those of M-protein levels in almost all cases, which means ASO-PCR kinetics are more sensitive to treatment effectiveness than M-protein kinetics. Interestingly, in one case (#3), ASO-PCR kinetics were almost the same as M-protein kinetics, which might indicate treatment refractoriness (Fig. 3B).

The patient-specific ASO primers could be designed in 25 cases, but not in 3 BJP-type cases or 2 IgG-type cases. One BM sample (#6) at diagnosis was insufficient, and no PCR product was detected. Two sets (#28 and #29) of the designed ASO primers failed to amplify the PCR products, and the standard curves were not drawn. In addition, the IgH/β-actin values for two patients (#12 and #19) were somewhat too low to evaluate. Therefore, we could design the PCR primers and quantify the ASO-PCR products in 20 of 30 BMMNC samples at diagnosis (Supplementary Table E2, online only, available at www.exphem.org).

Quantification of IgH gene fragments in cell-free DNA from the sera

We were able to detect patient-specific *IgH* DNA sequences in cell-free DNA extracted from the sera and quantify the ASO-PCR products. The ASO-PCR values in cell-free DNA were obtained in 18 cases at diagnosis, but not in 2 at diagnosis, 3 at 6mo, and 4 at 12mo. The changes in ASO-PCR levels in cell-free DNA over the clinical course differed from those of BMMNCs and PBMNCs (Table 2); myeloma cell-derived *IgH* DNA fragments in the sera stayed at similar levels and sometimes increased during treatment. Of importance, the sequences of the ASO-PCR products were identical to the originally designed sequence, suggesting that detection of the ASO-PCR products in cell-free DNA could reflect the persistence of myeloma cells somewhere in the body. Therefore, ASO-PCR using cell-free DNA is likely to have different significance from that using BMMNCs and PBMNCs.

ASO-PCR products for mRNA in $CD20^+CD38^-$ B-cells in bone marrow

The ASO-PCR products for $CD20^+CD38^-$ B-cells in BM were relatively low, but were clearly detected, in 17 cases at diagnosis, whereas we could not detect them in 5 cases. The IgH/β-actin levels in $CD20^+CD38^-$ B-cells in BM correlated with values for IgH/β-actin both in BMMNCs (Spearman's $\rho = 1.35$, $p < 0.001$; Table 2) and in PBMNCs (Spearman's $\rho = 1.09$, $p < 0.001$; data not shown). Thus, the evaluation of relatively earlier B-cell stages of myeloma cells seems to be of importance, including for the possible existence of MM clones in the $CD20^+CD38^-$ B-cell population in BM.

Discussion

We quantified the PCR products of patient-specific IgH VDJ fragments in mRNA from PBMNCs, BMMNCs, and BM $CD20^+CD38^-$ B-cells, as well as of cell-free DNA from the sera. All raw data are shown in a Supplementary Table E2 (online only, available at www.exphem.org).

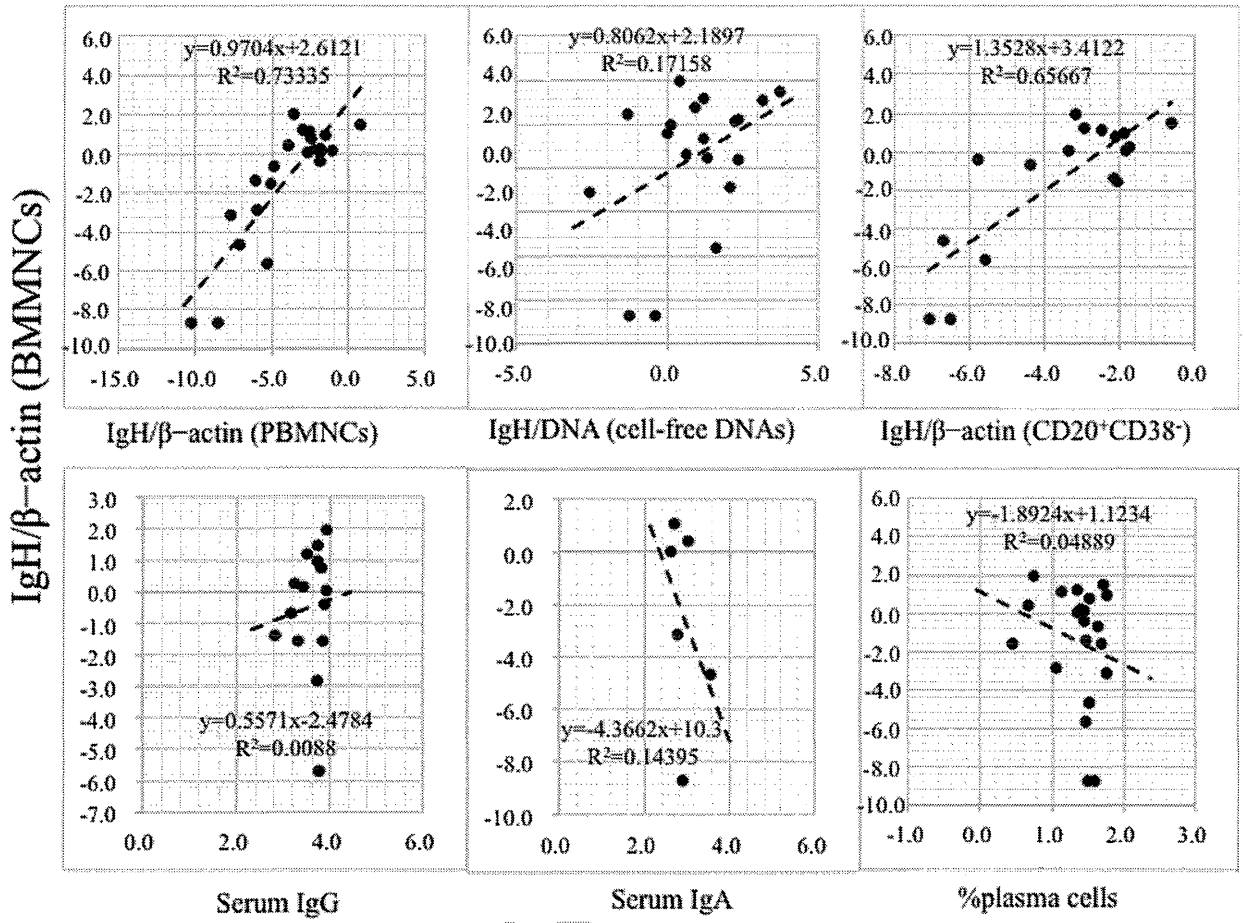

Figure 2. Univariate regression analysis of IgH/β-actin (BMMNCs) with several biomarkers at diagnosis. Log values of the indicated parameters were used for univariate regression analysis (R version 3.0.2). Closed circles represent the correlation of IgH/β-actin (BMMNCs) with IgH/β-actin (PBMNCs), IgH/β-actin ($CD20^+CD38^-$), ASO-PCR (cell-free DNA), M-protein, and percent plasma cells in each patient. Dashed lines represent the linear regression line.

The ASO-PCR is powerful and sensitive for detecting MRD [4,7–9,13–17], but it has some limitations. One problem is the cost of designing patient-specific ASO primers. The other is a failure to design primers because of the lack of clonal targets for amplification [8]. We applied this PCR method in 20 out of 30 cases, almost the same rate as previous reports [8]. In our cases, we often failed to design the patient's ASO primers and/or measure the PCR products in some BJP-type patients. We do not know the reason for this failure, which was not an issue in previous reports [4,8,14,15]. Although the sensitivity of ASO-PCR has been reported to be 10^{-5} to 10^{-6}, our results showed that gene amplification by ASO-PCR was highly dependent on the sequences of the designed primers. Thus, this restriction on designing primers is another problem. However, we could apply quantification of the ASO-PCR products to ample sources of clinical materials, such as PBMNCs, BM $CD20^+CD38^-$ B-cells, cell-free DNA, and BMMNCs.

There were statistically significant correlations in values for the ASO-PCR products between BMMNCs and PBMNCs, suggesting the possibility that clonogenic plasma cells or myeloma precursor cells may circulate in peripheral blood. In the previous reports [18,19], MM cells and clonogenic MM precursor B cells were detected in the peripheral blood. Thus, the ASO-PCR values in PBMNCs might reflect the sum of the circulating MM cells and clonogenic MM precursor B cells. Therefore, PBMNCs, rather than BMMNCs, have the potential to be evaluated for MRD in MM patients during and after treatment. Availability of PBMNCs for evaluation of MRD will relieve the pain of BM aspiration. Recently, next-generation DNA sequencing was shown to be more sensitive than ASO-PCR for detecting MRD in MM [20]. From our data, we anticipate that PBMNCs might be a good source for monitoring MRD even when the next-generation sequencing method is applied. Next-generation DNA sequencing could resolve some problems with ASO-PCR analysis, such as the high percentage of failure to design primers and the dependency of efficacy of amplification on primer sequences. However, the new technology is complicated and costs much more

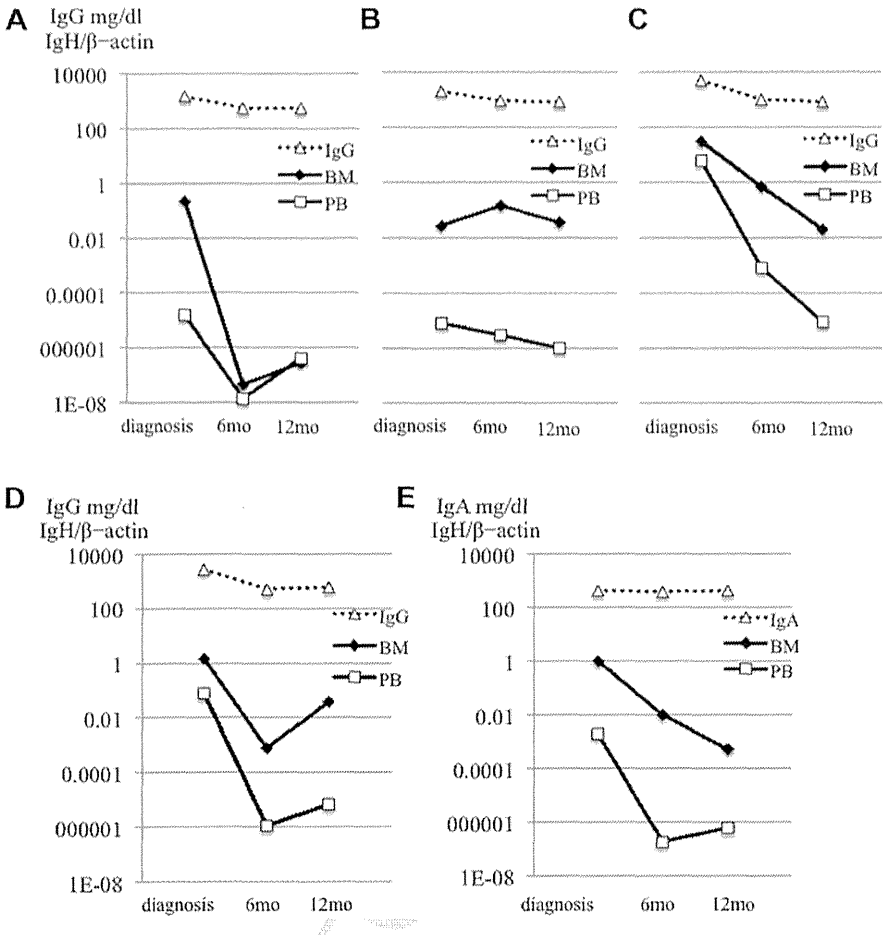

Figure 3. Representative cases, patients #1, #3, #8, #11, and #24. (**A**) Patient #1 was a 72-year-old male diagnosed with IgG κ–type MM without abnormal fluorescence in situ hybridization. (**B**) Patient #3 was a 56-year-old female diagnosed with IgG κ–type MM without abnormal fluorescence in situ hybridization. (**C**) Patient #8 was a 63-year-old female diagnosed with IgG κ–type MM with t(11;14). (**D**) Patient #11 was a 71-year-old male, with IgG κ–type MM without abnormal fluorescence in situ hybridization. (**E**) Patient #24 was a 49-year-old male diagnosed with IgA κ–type MM without abnormal fluorescence in situ hybridization. The IgH/β-actin values in the indicated clinical material, as well as serum IgG levels, are shown for diagnosis, 6mo, and 12mo.

than the ASO-PCR method. Therefore, the ASO-PCR method could still be useful for MRD evaluation, even in the era of next-generation DNA sequencing, and the method of analyzing MRD should be determined on a case-by-case basis.

We detected and quantified the ASO-PCR products of cell-free DNA from the sera. Thus, the presence of small amounts of circulating nucleic acids in plasma and serum might be important [21]. Reports that the amount of circulating nucleic acids in plasma and serum is significantly increased in cancer patients have aroused great interest [21,22]. In recent years, high levels of circulating cell-free DNA have been associated with cancer diagnosis and prognosis, and cell-free DNA has become a potential candidate biomarker for tumor detection [22]. The release of DNA from tumor cells can occur through various cell-physiologic events, such as apoptosis, necrosis, and secretion. However, the physiology and rate of release is still not well understood [23]. To the best of our knowledge, the current work describes for the first time that *IgH* DNA fragments from MM cells circulate in the sera. The presence of *IgH* DNA fragments from MM cells in the sera may reflect tumor fragments and/or sensitivity to treatment. The ASO-PCR values in the cell-free DNA from sera did not correlate with those in both BMMNCs and PBMNCs, which suggested the possibility of the different clinical meanings among those values. For example, the detection of the ASO-PCR products in cell-free DNA may mean that MM cells remain somewhere in the body beyond BM aspiration sites. To clarify the clinical significance of the circulating MM-related DNA fragments, such as their relationship with prognosis, a longer follow-up of MM patients in this cohort will be needed.

It has been postulated that so-called myeloma stem cells might be responsible for tumor initiation and relapse, but their unequivocal identification remains to be made [24–26]. We analyzed the $CD20^{+}CD38^{-}$ B-cell fraction because anti-CD20 antibodies are now available as a tool

of clinical treatment of B-cell malignancies. We detected and quantified ASO-PCR products of $CD20^{+}CD38^{-}$ B cells in BM at diagnosis in approximately three quarters of the patients examined. There were no obvious differences of the clinical features between the patients with and without detection of the ASO-PCR products in $CD20^{+}CD38^{-}$ B cells. Furthermore, the values for the ASO-PCR products of $CD20^{+}CD38^{-}$ B cells showed statistically significant correlations with those in both BMMNCs and PBMNCs. These results indicated that clonogenic MM cells might exist, not only in the $CD20^{-}CD38^{high}$ plasma cell fraction, but also in the $CD20^{+}CD38^{-}$ B-cell fraction, which might be another target of clonogenic MM precursor B cells to be treated by the different treatment strategies. These clonogenic B cells may circulate in peripheral blood and are in part identical to the clonal B-cell excess identified in our previous report [27]. Thus, we could consider treatment strategies to include anti-CD20 antibodies against the clonogenic $CD20^{+}CD38^{-}$ B-cell population.

This study is the first, to our knowledge, to measure quantitative ASO-PCR values in mRNA from various clinical materials of MM patients. This method may be useful for detecting MRD in patients with MM as well as for clarifying the pathogenesis of MM.

Uncited reference

[12].

Acknowledgments

We thank Noriko Kobayashi, Yoshiyasu Ogawa, and Masaaki Kihara at LSI Medience Corporation for their assistance with ASO primer design. This work was supported in part by medical Q6 research funding from the Mitsui Life Social Welfare Foundation.

Conflict of interest disclosure

Q7 H Sata, H Shibayama, K Fukushima, J Fujita, S Ezoe, S Tadokoro, T Maeda, M Mizuki, K Oritani, and Y Kanakura received research Q8 funding from Celgen, Janssen, and Takeda. The other authors Q9 declare no financial interest/relationships with financial interest relating to the topic of this article.

Q10 References

1. Gay F, Larocca A, Wijermans P, et al. Complete response correlates with long-term progression-free and overall survival in elderly myeloma treated with novel agents: analysis of 1175 patients. Blood. 2011;117:3025–3031.
2. Chanan-Khan AA, Giralt S. Importance of achieving a complete response in multiple myeloma, and the impact of novel agents. J Clin Oncol. 2010;28:2612–2624.
3. Watanabe R, Tokuhira M, Kizaki M. Current approaches for the treatment of multiple myeloma. Int J Hematol. 2013;97:333–344.
4. Takamatsu H, Ogawa Y, Kobayashi N, et al. Detection of minimal residual disease in patients with multiple myeloma using clonotype-specific PCR primers designed from DNA extracted from archival bone marrow slides. Exp Hematol. 2013;41:894–902.
5. Ferrero S, Drandi D, Mantoan B, Ghione P, Omedè P, Ladetto M. Minimal residual disease detection in lymphoma and multiple myeloma: impact on therapeutic paradigms. Hematol Oncol. 2011; 29:167–176.
6. Harousseau JL, Attal M, Avet-Loiseau H. The role of complete response in multiple myeloma. Blood. 2009;114:3139–3146.
7. Novella E, Giaretta I, Elice F, et al. Fluorescent polymerase chain reaction and capillary electrophoresis for IgH rearrangement and minimal residual disease evaluation in multiple myeloma. Haematologica. 2002;87:1157–1164.
8. Sarasquete ME, García-Sanz R, González D, et al. Minimal residual disease monitoring in multiple myeloma: a comparison between allelic-specific oligonucleotide real-time quantitative polymerase chain reaction and flow cytometry. Haematologica. 2005; 90:1365–1372.
9. Ladetto M, Donovan JW, Harig S, et al. Real-Time polymerase chain reaction of immunoglobulin rearrangements for quantitative evaluation of minimal residual disease in multiple myeloma. Biol Blood Marrow Transplant. 2000;6:241–253.
10. Durie BGM, Harousseau JL, Miguel JS, et al. International uniform response criteria for multiple myeloma. Leukemia. 2006;20: 1467–1473.
11. van Dongen JJM, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17:2257–2317.
12. Greipp PR, San Miguel J, Durie BGM, et al. International staging system for multiple myeloma. J Clin Oncol. 2005;23:3412–3420.
13. Korthals M, Sehnke N, Kronenwett R, et al. Molecular monitoring of minimal residual disease in the peripheral blood of patients with multiple myeloma. Biol Blood Marrow Transplant. 2013;19:1109–1115.
14. Billadeau D, Prosper F, Verfaillie C, Weisdorf D, Van Ness B. Sequential analysis of bone marrow and peripheral blood after stem cell transplant for myeloma shows disparate tumor involvement. Leukemia. 1997;11:1565–1570.
15. Cremer FW, Ehrbrecht E, Kiel K, et al. Evaluation of the kinetics of the bone marrow tumor load in the course of sequential high-dose therapy assessed by quantitative PCR as a predictive parameter in patients with multiple myeloma. Bone Marrow Transplant. 2000;26:851–858.
16. Cremer FW, Kiel K, Wallmeier M, Goldschmidt H, Moos MA. Quantitative PCR assay for the detection of low amounts of malignant cells in multiple myeloma. Ann Oncol. 1997;8:633–636.
17. Fenk R, Haas R, Kronenwett R. Molecular monitoring of minimal residual disease in patients with multiple myeloma. Hematology. 2004;9:17–33.
18. Paiva B, Perez-Andres M, Vidrials MB, Almeida J, de las Heras N, Mateos MV. Competition between clonal plasma cells and normal cells for potentially overlapping bone marrow niches is associated with a progressively altered cellular distribution in MGUS vs myeloma. Leukemia. 2011;25:697–706.
19. Kiel K, Cremer FW, Rottenburger C, et al. Analysis of circulating tumor cells in patients with multiple myeloma during the course of high-dose therapy with peripheral blood stem cell transplantation. Bone Marrow Transplant. 1999;23:1019–1027.
20. Martínez-López J, Lahuerta JJ, Pepin F, et al. Prognostic value of deep sequencing method for minimal residual disease detection in multiple myeloma. Blood. 2014;123:3073–3079.
21. González-Masiá JA, García-Olmo D, García-Olmo DC. Circulating nucleic acids in plasma and serum (CNAPS): applications in oncology. Onco Targets Ther. 2013;6:819–832.
22. Zane M, Agostini M, Enzo MV, et al. Circulating cell-free DNA, SLC5A8 and SLC26A4 hypermethylation, BRAF(V600E): A

noninvasive tool panel for early detection of thyroid cancer. Biomed Pharmacother. 2013;67:723–730.
23. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11: 426–437.
24. Paíno T, Ocio EM, Paiva B, et al. CD20 positive cells are undetectable in the majority of multiple myeloma cell lines and are not associated with a cancer stem cell phenotype. Haematologica. 2012;97: 1110–1114.
25. Kellner J, Liu B, Kang Y, Li Z. Fact or fiction-identifying the elusive multiple myeloma stem cell. J Hematol Oncol. 2013;6:91.
26. Matsui W, Wang Q, Barber JP, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer Res. 2008;68:190–197.
27. Oritani K, Katagiri S, Tominaga N, et al. Aberrant expression of immunoglobulin light chain isotype in B lymphocytes from patients with monoclonal gammopathies:isotypic discordance and clonal B-cell excess. Br J Haematol. 1990;75:10–15.

Supplementary Table E1. Sequences of specific IgH VDJ region primers

Patient number	*MM-MRD-5′* sequence (5′ to 3′)	Amplicon size
1	GTGCGAGACGGGTGGGGGC	74 bp
2	GCGAGGTCGGGATACTTGAA	91 bp
3	TATAGCAGCCTGGCGAAT	70 bp
4	TTATTGTGCGAGTAGCCCCTAT	91 bp
5	CGAACCTCCAGAATGGGCTA	84 bp
6	AGACACGGGCCGATTGATT	84 bp
7	Not designed	
8	GAAGGGGATGGCTGCAATTA	74 bp
9	CGCGGGATTTCGATATTTTG	73 bp
10	GTGCTAGAGCCGGGGGTATT	61 bp
11	CGACTACATTTGGGCGAACTT	61 bp
12	AGCTGGCCATTGAAGCCTTA	46 bp
13	Not designed	
14	AAAAGATTACTATGAGGACGGTAT	70 bp
15	CTGTGCACGGATGAGTGGTT	82 bp
16	AGCAGCAACTGGCCTTTTTG	64 bp
17	TGCGAGCATCCCCTAATAAAA	113 bp
18	Not designed	
19	GATTCCGGGAGATCATTCTGA	79 bp
20	Not designed	
21	AAAGATAACGGCGGTGGCTA	78 bp
22	GGGTCTGGTGGTGTATGACGA	79 bp
23	CGAGAGAATATCAGGTCCACTTCC	77 bp
24	GATAGCAGCACTTATGACCCTCAA	75 bp
25	GGTGTTCCCCTCTCCTACTTCT	75 bp
26	TGGAAGTAGAGATAAGCGAGGA	76 bp
27	Not designed	
28	CACACATCGGCAGGGATG	80 bp
29	TGCGAAGAATTTCAACACCTG	85 bp
30	GTGCGAGAGTGGGGACCA	77 bp

Supplementary Table E2. All patient data

Pt number	Type	Primer design	Standard curve	Best response	IgH/β−actin in BMMNCs			IgH/β−actin in PBMNCs			Cell-free DNA in serum (copy/μgDNA)			IgH/β−actin in CD20+CD38- B cells in BM at diagnosis
					Diagnosis	6mo	12mo	Diagnosis	6mo	12mo	Diagnosis	6mo	12mo	
1	IgG	○	○	sCR	0.211339508	0.000000046	0.000000288	0.000014798	0.000000014	0.000000414	17.3502994	7.519572954	5.536337209	0.000039098
2	IgA	○	○	SD	2.839170836	NA	0.172292123	0.000108994	0.000000759	0.000000759	0.049782609	NA	0.466533865	ND
3	IgG	○	○	VGPR	0.027021138	0.138307552	0.03371831	0.000008010	0.000002997	0.000000993	237.8306878	390.6158358	381.7610063	ND
4	IgG	○	○	PR	0.029075242	0.004292972	0.014248753	NA	0.000001178	0.000000307	20.09146341	NC	38.71717172	0.00816969
5	IgA	○	○	PR	0.000742143	0.000000006	1.26556495	0.000000018	0.000000000	0.000002193	0.002386266	0.001236038	0.076	ND
6	IgG	○		PR	NA	0.11418559	0.916622968	0.000325256	0.000003784	0.000059005	4.224358974	9.142222222	1.681428571	NA
7	BJP	×												
8	IgG	○	○	VGPR	30.63051702	0.72168906	0.019994407	6.430276168	0.000764185	0.000008976	6023.404255	246.2149533	ND	0.233208588
9	IgG	○	○	VGPR	0.001424515	0.000063528	0.000200712	0.000000942	ND	ND	126.4191419	85.65217391	ND	ND
10	IgG	○	○	VGPR	0.000002155	0.000001435	0.000000195	0.000004380	0.000000492	0.000000093	43.0548926	84.23976608	35.49418605	0.000002454
11	IgG	○	○	VGPR	1.491101115	0.000782903	0.036973655	0.076746269	0.000001118	0.000006880	185.7083333	425.9223301	ND	ND
12	IgA	○	○	sCR	0.000000002	0.000000025	0.000000007	0.000000003	0.000000007	0.000000005	0.393877551	1.608027523	0.234576271	0.000000078
13	BJP	×												
14	IgG	○	○	PR	1.072881015	0.244382022	0.002578132	0.014568690	0.000000179	0.000000701	ND	15.41666667	10.67514677	0.014545455
15	IgA	○	○	CR	12.62883236	3.288605697	NA	0.002600774	0.000074976	NA	1444.244604	ND	NA	0.00337657
16	IgG	○	○	SD	15.79178886	6.640151515	NA	0.001035784	0.000309120	0.001436230	16.41348774	9.837150127	9.93287037	0.001113991
17	IgA	○	○	VGPR	0.000022392	0.000569374	0.000000251	0.000000071	0.000000737	0.000000002	NA	5.56	141.2053571	0.000000194
18	IgG	×												
19	BJP	○	○	sCR	0.000000002	0	0	0.000000000	0.000000000	0.000000000	0.050775862	0.083726415	7.080033003	0.000000285
20	IgG	×												
21	IgG	○	○	PR	0.392692146	0.016889833	0.029146746	0.012726930	0.064271917	0.010137971	1.045945946	0.216615854	124.1588785	0.000001609
22	IgG	○	○	SD	0.041076516	0.076784823	NA	0.000000692	0.000005332	NA	4.535031847	2.820276498	NA	0.006679562
23	IgG	○	○	PR	99.35720845	50.42265427	0.062986831	0.000288493	0.000000265	0.000007738	2.392307692	4.430451128	135.1973684	0.000688948
24	IgA	○	○	VGPR	1.035455861	0.009744824	0.000498450	0.002006000	0.000000189	0.000000633	1.290810811	2.021621622	71.5648855	0.000440181
25	IgG	○	○	VGPR	9.423288173	0.007192324	0.020667539	0.030932919	ND	0.000001174	ND	ND	ND	0.013472104
26	IgG	○	○	SD	5.679324895	NA	1.048479583	0.003660095	NA	0.004949617	8.048387097		29.76973684	0.007968837
27	BJP	×												
28	IgA	○	×											
29	BJP	○	×											
30	IgG	○	○	VGPR	1.732207479	0.000399577	0.000924577	0.014009057	0.000000027	ND	223.3333333	123.5185185	4.05	0.018027901

NA = Not available; *ND* = not detected.

EXPHEM3224_proof ■ 11-2-2015 16-53-32

特　集
血液分野の最新情報2015 —バイオロジーを中心に（赤血球系疾患）—

PNH患者におけるC5遺伝子多型

西村純一，　金倉　譲

Eculizumab投与を受けた日本人PNH患者345例中11例が不応症であったが，この分子的機構は不明であった。全11例で共通のC5のミスセンスヘテロ接合性変異c.2654 G>Aを認め，p.Arg885Hisが予測された。患者の変異保有率（3.2%）は，健常人の保有率（3.5%）と同程度で，漢民族においても確認された。この変異型C5は野生型C5とともに*in vitro*溶血を引き起こしたが，野生型C5のみがeculizumabと結合し，eculizumabによる阻害を受けた。この*in vitro*溶血は，eculizumabとは異なるC5部位に結合する抗体N19-8を用いると，野生型C5，変異型C5ともに完全に阻害された。Arg885His変異を伴うC5遺伝子多型の機能的特性は，eculizumabによる阻害を受けず，この変異を有する患者のeculizumab不応症を説明し得ることが示された。（臨床血液 **56**（2）：103〜110，2015）

Key words：Paroxysmal nocturnal hemoglobinuria, Complement mediated intravascular hemolysis, Eculizumab, Gene polymorphism

はじめに

発作性夜間ヘモグロビン尿症（paroxysmal nocturnal hemoglobinuria, PNH）の主要症状である補体介在性溶血に対する治療薬として，補体蛋白C5を標的とするヒト化モノクローナル抗体であるeculizumabが開発され，長期使用における有効性と安全性のデータが海外[1]と日本[2]から相次いで報告された。さらに，小児患者においても，成人と同様の有効性と安全性を示すデータが公表された[3]。その一方で，溶血の指標である血清LDH値が全く低下しない不応症が，日本人PNH患者の一部の集団に見いだされ問題となっていたが，その分子機構が解明された[4]。本稿ではこの分子機構を中心に概説するとともに，C1[5]やC3[6]といったC5より上流の分子を標的にした治療薬開発の動きも加速しつつあり，それら候補薬剤の可能性についても言及したい。

PNHの病態

1. 補体介在性血管内溶血

PNH患者の血液細胞では，補体制御因子であるCD55（decay-accelerating factor, DAF）とCD59（membrane inhibitor of reactive lysis, MIRL）の発現が欠損または低下している。CD55はC3/C5転換酵素の崩壊を促進することによって補体活性化経路の前半の段階を調節するのに対し[7]，CD59はC9に作用して膜侵襲複合体（membrane attack complex, MAC）の形成を阻害する[8,9]。PNH患者では，平常でもわずかな補体活性化による持続的な溶血がみられるが，感染症，睡眠，手術，妊娠，鉄剤投与など様々な誘因により強い補体活性化が起こると，短時間で大量溶血（溶血発作）をきたす。これら誘因の中でも，臨床的にしばしば問題となるのは感染症である。PNH患者は，補体介在性の血管内溶血，骨髄不全および血栓症を3大症状とするが，他にも，腹痛，嚥下障害，男性機能不全などの多彩な症状を示す。溶血により血漿中に放出された遊離ヘモグロビンが，Nitric Oxide（NO）を強力に捕縛し，NOの作用を阻害する結果，腹痛，嚥下障害，男性機能不全，血栓症などの症状を誘発，増幅する機序が明らかになった[10]。

2. PNHクローンの拡大機序

PNHは，一つ（または数個）の造血幹細胞の*phosphatidylinositol glycan class A*（*PIGA*）遺伝子に後天的変異が起こり，その変異細胞がクローン性に拡大する造血幹細胞疾患である[11]。*PIGA*遺伝子変異によりglycosylphosphatidylinositolアンカー型蛋白（GPI-AP）の合成障害をきたし，CD59やCD55などのGPIアンカー型補

大阪大学大学院医学系研究科血液・腫瘍内科