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Original Research Article

Quantification of bone marrow plasma cell infiltration in multiple myeloma: usefulness of bone marrow aspirate clot with CD138 immunohistochemistry

Kosei Matsue[1]*, Yuya Matsue[2], Kaoru Kumata[3], Yoshiaki Usui[1], Yasuhito Suehara[1], Kota Fukumoto[1], Manabu Fujisawa[1], Kentaro Narita[1] and Masami Takeuchi[1]

[1]*Division of Hematology/Oncology, Department of Internal Medicine, Kameda Medical Center, Kamogawa-shi, Chiba, Japan*
[2]*Department of Cardiology, University Medical Center Groninge, Groninge, The Netherlands*
[3]*Department of Clinical Pathology, Kameda Medical Center, Kamogawa-shi, Chiba, Japan*

**Correspondence to: Kosei Matsue, MD, PhD, Division of Hematology/Oncology, Department of Medicine, Kameda Medical Center, 929 Higashi-chou, Kamogawa-shi, Chiba 296-8602, Japan.*
E-mail: koseimatsue@gmail.com

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Abstract

Accurate quantification of plasma cells (PCs) in bone marrow (BM) is critical for diagnosis and assessment of treatment response in patients with multiple myeloma (MM). We compared the % of BM PC quantified by 250 cell differential count on May–Giemsa-stained BM smears, by counting 500 – 2500 cells in 2 – 5 representative microscopy fields in CD138-immunostained BM clot and biopsy sections, and CD38/CD45/CD138 gated BM PCs on flow cytometry (FCM) in 150 sets of BM samples from 120 patients. Percentages of PC were significantly correlated between BM biopsy and clot, and between smear and FCM ($r = 0.96$, 0.93, respectively). However, quantification by smear and FCM significantly underestimated the PC compared to biopsy or clot, and the degree of underestimation increased with blood dilution. FCM consistently showed lower % of PC compared to aspirate smears. Fifty-nine of 103 patients with M-protein level < 3000 mg/dL in serum or 500 mg/24 h in urine and diagnosed with monoclonal gammopathy of undetermined significance (MGUS) based on smear alone were reclassified as smoldering MM when reassessed using CD138-stained biopsy/clot sections. Among the 72 patients with sMM diagnosed by BM biopsy and/clot, three patients (4.2%) had extensive BM infiltration of PC (≥ 60%) and required treatment. Our data clearly showed the necessity of CD138 immunostaining of BM biopsy/clot specimens for correct diagnosis of MM and related disorders.

Keywords: **multiple myeloma; CD 138 immunostaining; bone marrow biopsy; aspirate smear; plasma cell quantification; flow cytometry**

Introduction

Estimation of plasma cell (PC) infiltrates in bone marrow (BM) is integral to the diagnosis and monitoring of patients with suspected PC dyscrasias. The recently updated International Myeloma Working Group (IMWG) criteria [1] for diagnosis of multiple myeloma (MM) define MM as >10% BM PCs as the highest percentage on aspirate or trephine biopsy sections. In addition, patients with >60% clonal BM PCs should be treated as asymptomatic MM, even if they have no MM-related organ damage [1,2]. The IMWG defines complete response as < 5% clonal PCs in BM. Therefore, it is critical to assess the PC infiltrates correctly. Traditionally, quantification of BM PCs has been performed by differential counting of May–Giemsa-stained aspirate smears. However, BM aspirates are often affected by blood dilution and age- or therapy-related BM hypoplasia. Increased marrow fibrosis, which is seen in approximately 10% – 30% of myeloma patients, also affects the quality of aspirated samples [3]. A combination of trephine biopsy sections of BM with CD138 (syndecan-1) immunostaining has been studied as an alternative for identification of PCs [4,5]. CD138 is expressed in mature epithelial cells and some non-hematopoietic neoplasms, including various carcinomas and melanomas, but is only expressed in normal and malignant PCs among hematopoietic cells and can therefore be used as a reliable marker for identification of PCs [6]. Previous studies consistently showed a higher percentage of PCs in trephine sections compared to BM aspirate smears [4,7,8]. Nevertheless, the IMWG criteria still allow use of aspirate smears as an alternative to core biopsy specimens because core biopsy cannot always be readily performed in daily practice. In addition, repeated biopsies are not feasible because of BM

scarring or destruction of cortical bone. Trephine BM biopsy with CD138 immunostaining has not been widely adopted in clinical practice as a consequence of these shortcomings, and many institutions around the world still determine BM PC percentage based on aspirate smears alone.

As clotted sections of BM aspirates contain hematopoietic elements that are not diluted by peripheral blood, analysis of BM clot sections could be a promising alternative to BM trephine biopsy. This study was performed to compare the accuracy of different methods for quantification of PC [BM aspirate clot, BM aspirate smear, and multicolor flow cytometry (FCM)], as the BM PC percentage is critical to guide subsequent decision-making in the diagnosis and treatment of monoclonal gammopathy of undetermined significance (MGUS) and MM. Further, to clarify the clinical relevance of quantifying BM PCs by CD138-stained BM aspirate clots, we examined 116 patients with monoclonal gammopathy who did not fulfill the criteria of smoldering MM according to the PC percentage of BM on aspirate smears and aspirate clots stained with anti-CD138 monoclonal antibody.

Patients and methods

A total of 150 BM samples from 100 patients with MGUS and MM, at diagnosis or following therapy, in whom simultaneous BM aspirate, biopsy, and FCM analysis were performed between April 2011 and December 2015 were selected from the database of Hematology/Oncology, Kameda Medical Center. Samples of 3 – 5 mL of BM aspirate were taken for smears, clot sections, and FCM analysis. The aspirate smears were stained with May–Giemsa, and 2 mL of heparinized aspirate was subjected to FCM analysis, simultaneously. The rest of the aspirated marrow was left in the syringe for 20 – 30 min without mixing until it clotted. Clotted aspirate was then fixed in 5% formalin and subjected to staining with hematoxylin and eosin (H–E) and CD138 immunohistochemistry. BM biopsy was performed with an 11-gauge trephine biopsy needle from the iliac crest and fixed in 5% of formalin. The samples were decalcified, embedded in paraffin, and then sections 2 – 3 μm thick were subjected to H–E, silver staining, Congo red staining, and CD138 immunohistochemistry.

The PC percentages were calculated from 250 nucleated cell counts on BM aspirate smears by three qualified laboratory specialists and hematologists. For CD138 immunohistochemical quantification of PCs in BM biopsies and clot sections, they were initially assessed at low magnification, and 2 – 5 representative fields or hematopoietic elements were selected for counting. At least 500 cells were counted at intermediate magnification (×200) (Figure 1).

FCM analysis was performed on BM aspirate samples (Navios flow cytometer; Beckman-Coulter, USA), and the cells were analyzed using Kaluza software (Beckman-Coulter) as reported previously [9]. PC identification required at least two markers, i.e. CD38 and either CD45 or CD138. The proportions of CD38-positive and either CD45- or CD138-positive cells were calculated relative to the total nucleated cell population, including nucleated red cells.

To investigate the clinical relevance of CD138 immunohistochemical staining in the differentiation of MGUS and MM, BM slides from 116 untreated patients with M-protein level < 3000 mg/dL in serum or 500 mg/day in urine and with no organ damage were assessed with regard to diagnosis according to the percentage of BM PCs.

Written informed consent was obtained as well as Institutional Review Board approval from Kameda General Hospital in accordance with the Declaration of Helsinki.

Statistical analysis

We considered CD138-positive PC count in BM biopsy sections as the gold standard method to quantify PC.

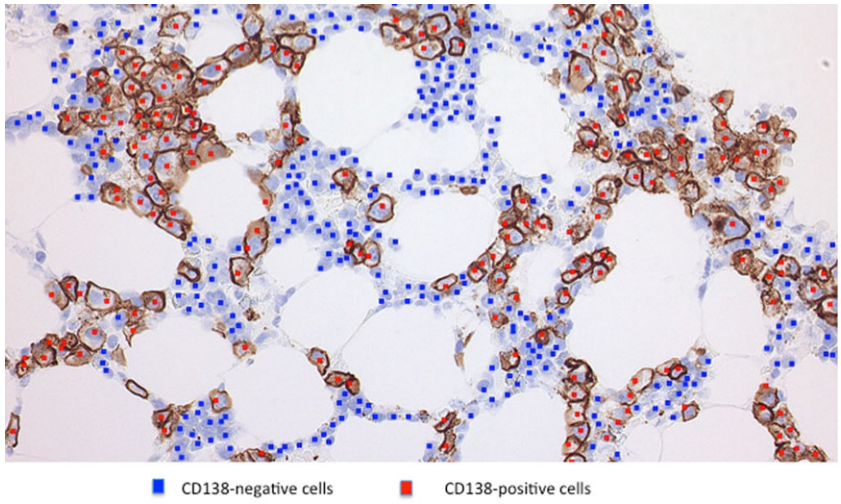

Figure 1. Quantification of bone marrow plasma cells by CD138 immunohistochemistry

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Pearson's correlation was used to evaluate the correlation between percentage of PC measured by BM biopsy and BM clot, BM biopsy and BM smear, and BM biopsy and FCM. Bland–Altman plots of differences versus means coupled with analysis of the slope of the regression line were used to evaluate the presence of fixed and/or proportional bias [10,11]. To evaluate fixed bias between BM biopsy and each measurement method, 95% confidence intervals (95% CI) of mean difference were estimated by bootstrapping the data 2000 times. The 95% CI of the differences should not differ significantly from zero in the absence of fixed bias. The regression between the differences and corresponding means was plotted, and a slope different from zero was used to indicate the presence of proportional bias [10]. The interclass correlation coefficient (ICC) was also calculated to examine the agreement between measurement methods in the same specimen. A two-tailed P value < 0.05 was considered statistically significant. Statistical analyses were performed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria). ISBN 3-900051-07-0, URL http://www.R-project.org.

Results

PC localization pattern in trephine biopsy sections and BM aspirate clots

In BM biopsy or clot sections, CD138 immunohistochemical staining could be divided into four morphological patterns: interstitial, micro-aggregated, focal, and diffuse (Figure 2). Only sections with interstitial or aggregated patterns were included in this study, as PCs with large nodular and diffuse patterns were considered inappropriate for quantification because of the high variability of localization of PCs on the slides. Cells with Golgi staining or nonspecific cytoplasmic staining were excluded from the calculation; only membranous stained cells were considered as positive [5].

Because of the difficulty of manually counting cells with large nodular pattern and diffuse pattern, 18 samples with nodular or diffuse patterns were excluded in the current study. Therefore, 132 sets of samples were included in the analysis.

PC enumeration using different methodologies

Figure 3 shows scatter plots comparing BM PC percentages obtained from BM biopsies, aspirate clots, aspirate smears, and FCM. Median levels of measured BM biopsy, BM clot, BM smear, and MFC were 13.3 (IQR: 6.7 – 36.2)%, 12.8 (IQR: 6.8 – 31.9)%, 3.7 (IQR: 1.2 – 10.0)%, and 2.4 (IQR: 0.8 – 6.2)%, respectively. There were significant correlations between BM biopsy and the three other measurement methods (Figure 3), and BM clot showed the strongest correlation with BM biopsy ($r = 0.94$). We arbitrarily defined BM dilution by the nucleated cell count cut-off of $40 \times 10^3/\mu L$ to evaluate interaction of dilution with the association between these measurement methods.

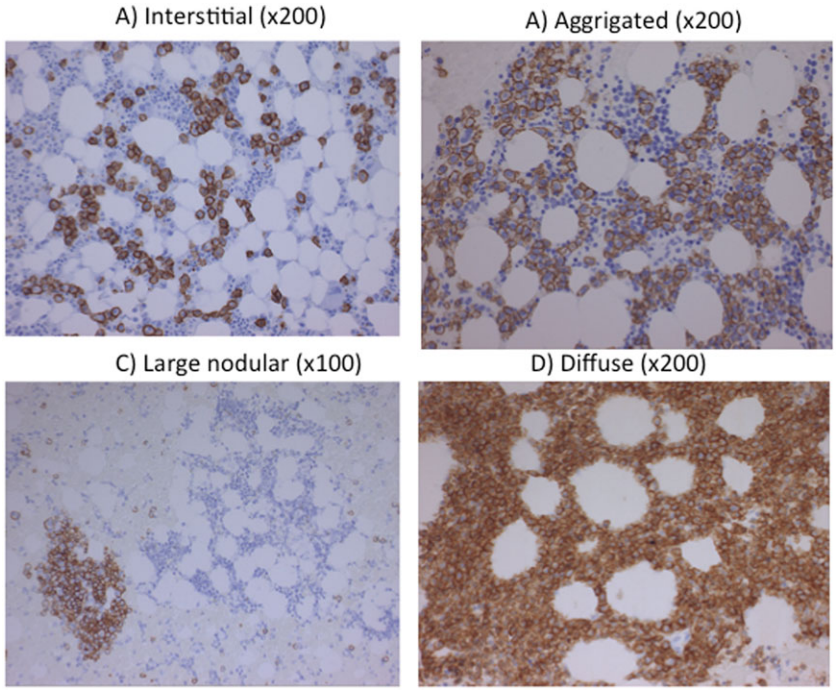

Figure 2. Bone marrow plasma cell infiltration pattern by CD138 immunostaining. A) Interstitial B) Aggregated C) Large nodular D) Diffuse

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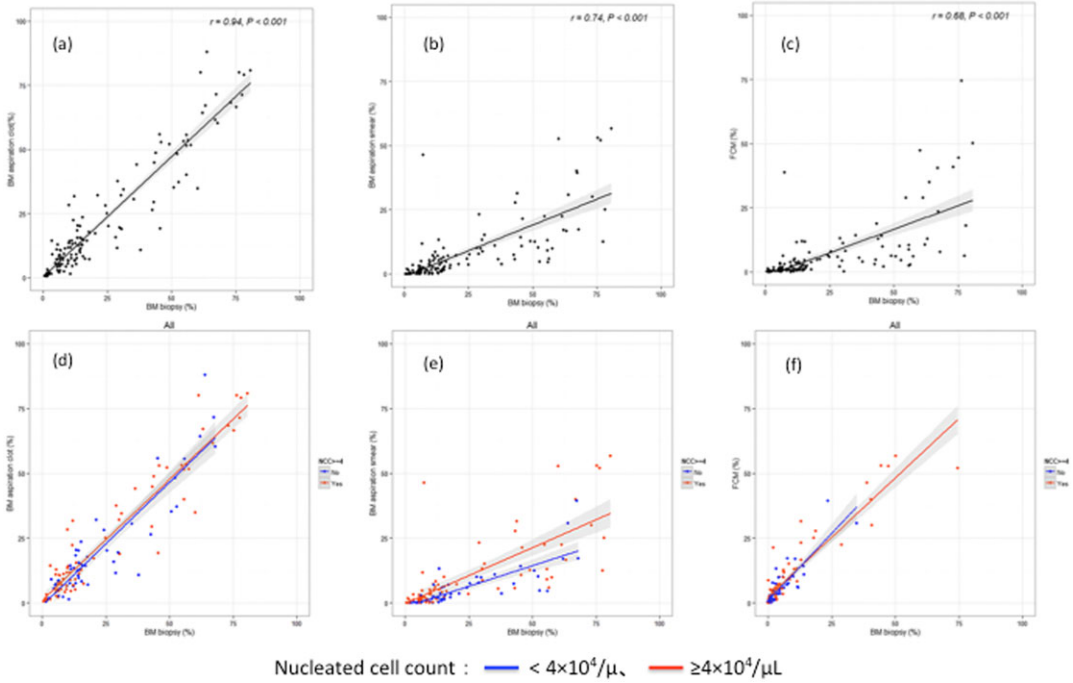

Figure 3. Scatter plots of bone marrow plasma cell percentages obtained from bone marrow biopsies, aspirate clots, aspirate smears, and flow cytometry (FCM). Black lines indicate the regression lines of whole data, and blue and red lines indicate regression lines for samples with nucleated cell count (NCC) $< 4 \times 10^4/\mu L$ and $\geq 4 \times 10^4/\mu L$, respectively

In correlation analysis, BM biopsy and BM clot showed significant and very strong correlations in both NCC $\geq 40 \times 10^3/\mu L$ and NCC $< 40 \times 10^3/\mu L$ ($r = 0.95$ and $r = 0.91$, respectively). With regard to ICC, BM clot showed very high ICC to BM biopsy (0.97, 95%CI: 0.95 – 0.98) but neither BM smear (0.63, 95%CI: 0.02 – 0.83) nor FCM (0.57, 95%CI: –0.49 – 0.79). Bland–Altman plots showed that BM biopsy agreed well with BM clot but not with BM smear or FCM (Figure 4). There was a significant fixed bias between BM biopsy and BM smear (mean difference = 15.0%, 95% CI: 12.4% – 17.5%) and between BM biopsy and BM FCM (mean difference = 16.4%, 95% CI: 13.8 – 19.4), indicating that both of these measurements significantly underestimated PCs compared to BM biopsy. However, there was no significant fixed bias between BM biopsy and BM clot (mean difference = 1.1% 95% CI: – 0.24 – 2.42). We also evaluated proportional bias by regressing the differences in values on means of values with linear regression analysis; significant proportional bias was found between BM biopsy and BM smear (regression coefficient = 0.672, $P < 0.001$) and BM biopsy and FCM (regression coefficient = 0.706, $P < 0.001$), but not between BM biopsy and BM clot (regression coefficient = −0.001, $P = 0.966$).

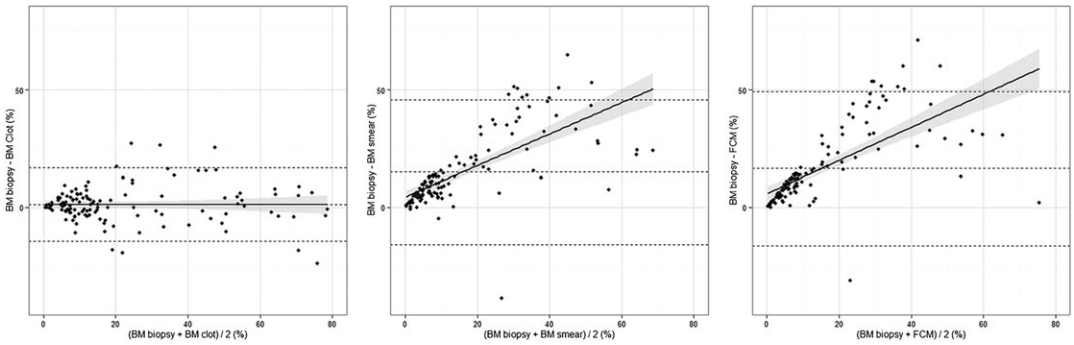

Figure 4. Bland–Altman plots of differences between BM biopsy and each measurement method (*y* axis) and means of BM biopsy and each measurement method (*x* axis). Linear regression lines with 95% confidence interval (shaded area) are provided

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Clinical implications of BM PC enumeration method (Figure 5)

As CD138 immunohistochemical staining consistently yielded a higher percentage of BM PCs than aspirate smear counting, it is possible that a considerable portion of patients diagnosed as MGUS by aspirate smear alone could be reclassified as smoldering MM (sMM) by CD138 immunostaining of BM aspirate clot or biopsy. We retrospectively reanalyzed the 116 consecutive patients with monoclonal gammopathy and < 3000 mg/dL or 500 mg/dL M-protein in serum or urine, respectively, at our hospital between April 2011 and December 2015. Among the 116 patients that met the above criteria, 13 patients (11%) were classified as smoldering myeloma as BM PCs exceeded 10% on aspirate smears. Among the remaining 103 patients, 59 patients (51%) showed >10% BM PCs by CD138 immunostaining and the remaining 44 patients (38%) were still classified as MGUS.

As the sMM patient with ≥60% of BM PC can be considered as symptomatic MM according to the updated IMWG criteria[1], we checked the % of BM PC in patients with sMM diagnosed by BM biopsy and/or clot. Among the 72 patients diagnosed as sMM, three patients (4.2%) had ≥60% of BM PC and considered as high risk sMM by BM biopsy and/or clot. Two of these patients showed extensive BM fibrosis. These patients were considered as having MM requiring therapy.

Discussion

BM PC quantification has traditionally been assessed by May–Giemsa staining of BM aspirates. However, enumeration of PCs on BM aspirate smears has several limitations, including dilution because of marrow fibrosis, hypoplasia, and patchy distribution of PCs within the BM. In addition, neoplastic PCs are often difficult to identify by morphology alone and are unevenly distributed on the smears on glass slides. Previous studies [1,3,8] showed consistently lower PC percentage in BM aspirate smears compared to biopsy. Our data also showed that PC counts on BM aspirate smears and FCM were significantly lower than those of BM biopsies or aspirate clots. PC counting by BM biopsy showed excellent correlations with aspirate clots. BM aspirate smears and FCM also showed good correlations, but both of these methods consistently underestimated the percentage of BM PCs compared to biopsies or clots stained with anti-CD138 monoclonal antibody. The percentage of BM PCs in aspirate clots did not affect BM nucleated cell counts above or below 40 000/μL; this could be explained as PC counting in BM aspirate clots was performed only on cells in BM hematopoietic elements, and did not include cells of the diluted blood. Therefore, we concluded that BM aspirate clots could be an alternative to biopsy specimens for quantification of BM PCs. This is important for clinical practice because BM biopsy is less feasible for repeated examinations compared to BM aspirate.

Significant discrepancies were often seen in the quantification of PCs between biopsy or clot and aspirate smears even when the percentage of BM PCs was high in our study. Marrow fibrosis has often been seen in patients with massive neoplastic PC burden that could be associated with poorly aspirated BM smears [3,12]. In light of the recent recommendation by the IMWG, this is very important from the clinical viewpoint as smoldering MM

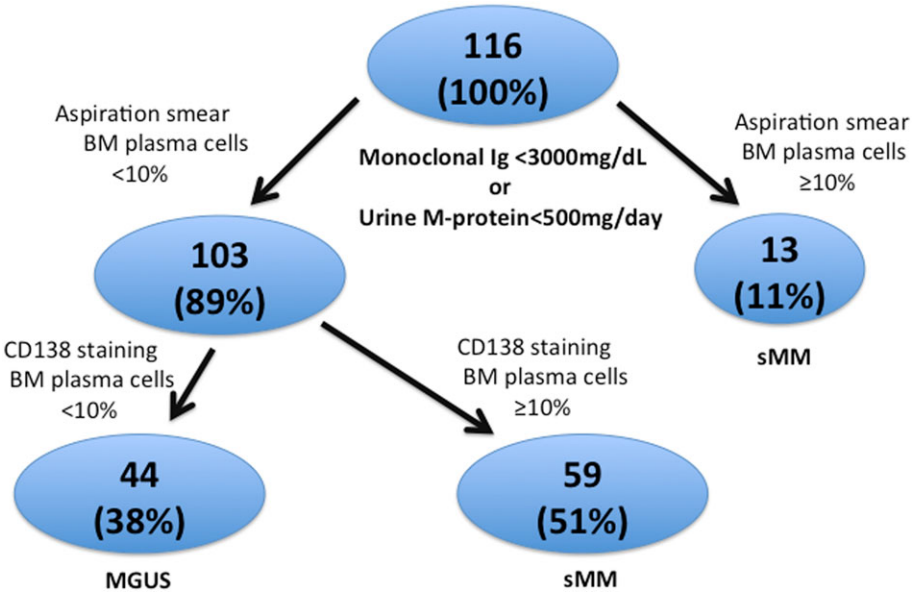

Figure 5. Analysis of patients with monoclonal gammopathy (MGUS) who did not meet the criteria of smoldering MM (sMM) according to bone marrow plasma cell infiltration

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(sMM) in patients with a high percentage of BM PCs (≥ 60%) should be treated as symptomatic myeloma [1,2]. Therefore, BM biopsy is strongly recommended for the initial evaluation of PC burden in patients with newly diagnosed MM with suspected high tumor burden. In addition, the biopsied samples should also be assessed for degree of fibrosis as well as quantification of BM PCs.

Consistent with previous observations [9,12,13], the percentage of PCs in BM smears was significantly correlated with FCM, although aspirate smears consistently gave a higher number of PCs over FCM. Biopsy and clot sections stained with anti-CD138 monoclonal antibody consistently showed a higher percentage of PCs compared to BM smears. These observations suggested that a diagnosis of MGUS by BM smear alone could misclassify the sMM as MGUS. Therefore, we reexamined 116 patients with low paraprotein levels (serum M-protein < 3000 mg/dL or urine M-protein < 500 mg/24 h). Thirteen patients had ≥10% BM PCs and were diagnosed as sMM by BM smear alone. Among the remaining 103 patients diagnosed as MGUS by BM smear alone, 59 patients were diagnosed as sMM by CD138 immunostaining of BM aspirate clots. These observations indicated that a considerable proportion of patients could be misclassified as MGUS by BM aspirate alone under the current diagnostic criteria. Similar observations were made by Ng et al. [8], who reported that a quarter of patients were potentially misclassified as having less extensive disease by aspirate smear alone as compared with concordant CD138 immunostained biopsy sections. Similarly, quantification of PC by BM aspirate alone could underestimate the PC of patients with sMM who had ≥60% BM PC and required treatment [1,2]. Three of 72 sMM patients (4.2%) had extensive BM infiltration (≥60%) of PC determined by BM biopsy/clot with CD138 immunostaining.

Our data confirm the previous observations and highlight the need for reconsidering the diagnostic criteria of percentage of BM PC. We strongly recommend the use of BM clot CD138 immunostaining as for diagnosis and response assessment of PC dyscrasias.

Conflict of interest

All authors have declared no conflict of interest.

Acknowledgement

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Authorship statement

KM designated and performed the study. KM and YM performed the statistical study and wrote a manuscript. KK performed the immunohistochemical study. YU, YS, KF, MF, KT, and MT took care of patients and performed the BM examinations.

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